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DEVELOPMENT OF MARINE SEDIMENT TOXICITY DATA FOR ORDNANCE COMPOUNDS AND TOXICITY IDENTIFICATION EVALUATION STUDIES AT SELECT NAVAL FACILITIES

An Investigation Conducted by

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August 2000

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To assess the potential impact of contamination by ordnance compounds and their contribution to environmental degradation in two areas of Puget Sound, Washington, an extensive study was undertaken. It included the generation of a toxicity database of ordnance compounds to marine organisms, and sediment toxicity and chemical analyses of 50 stations in the vicinity of the Jackson Park and Port Hadlock naval faciilties, Puget Sound, including Toxicity Identification Evaluation (TIE) studies at selected stations. Eight ordnance compounds of concern were selected and analyzed for toxicity with six different kinds of marine toxicity tests, which included five species and phyla, and nine endpoints. Toxicity tests and endpoints were: fertilization success and embryological development with the sea urchin *Arbacia punctulata*; zoospore germination, germling length and cell number with the green macro-alga *Ulva fasciata*; survival and reproductive success of the polychaete *Dinophilus gyrociliatus*; larvae survival with the redfish *Sciaenops ocellatus*; and survival of juveniles of the opossum shrimp *Mysidopsis bahia*. The overall toxicity, chemistry, and TIE test results indicate that ordnance compounds are not contaminants of concern at any of the sites studied in Puget Sound, Washington.

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EXECUTIVE SUMMARY

To assess the potential impact of contamination by ordnance compounds and their contribution to environmental degradation in two areas of Puget Sound, Washington, an extensive study was undertaken. It included the generation of a toxicity database of ordnance compounds to marine organisms, and sediment toxicity and chemical analyses of 50 stations in the vicinity of the Jackson Park and Port Hadlock naval facilities, Puget Sound, including Toxicity Identification Evaluation (TIE) studies at selected stations.

Eight ordnance compounds of concern (2,4-dinitrotoluene, 2,6-dinitrotoluene, 2,4,6-trinitrotoluene, 1,3-dinitrobenzene, 1,3,5-trinitrobenzene, tetryl, picric acid and royal demolition explosive) were selected and analyzed for toxicity with six different kinds of marine toxicity tests, which included five species and phyla, and nine endpoints. Toxicity tests and endpoints were: fertilization success and embryological development with the sea urchin *Arbacia punctulata*; zoospore germination, germling length and cell number with the green macro-alga *Ulva fasciata*; survival and reproductive success of the polychaete *Dinophilus gyrociliatus*; larvae survival with the redfish *Sciaenops ocellatus*; and survival of juveniles of the opossum shrimp *Mysidopsis bahia*.

The most sensitive toxicity tests overall were the macro-alga zoospore germination and the polychaete reproduction tests. The most toxic ordnance compounds overall were tetryl and 1,3,5-trinitrobenzene. These were also the most degradable ordnance compounds, oftentimes being reduced to very low levels or even zero at the end of the test exposure period. Among the dinitro- and trinitro-toluenes and benzenes, toxicity tended to increase with the level of nitrogenation. Picric acid and Royal Demolition Explosive (RDX) were the least toxic chemicals tested overall.

For the sediment survey and TIE study, 25 stations located at the Jackson Park site and 25 at the Port Hadlock site were selected for sediment porewater toxicity testing. Sediment porewater was pneumatically extracted and toxicity determined using the sea urchin (*A. punctulata*) fertilization and embryological development tests. The embryological development test was more sensitive than the fertilization test, and only one station at Jackson Park and six at the Port Hadlock site did not exhibit toxicity to the former. The sediments from eight of the most toxic stations at the Jackson Park site and from five stations at the Port Hadlock site were selected for chemical analyses for ordnance compounds, organochlorinated pesticides, polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), butyltins, and trace metals.

Concentrations of several contaminants exceeded threshold level sediment quality guidelines values (Long *et al.*, 1995; Mac Donald *et al.*, 1996). However, ordnance compounds were not detectable, except for picric acid which occurred in very low levels (≤ 1 mg/kg sediment dry weight). Based on toxicity and chemistry data, three adjacent stations, OB8, OB18 and OB22 (Figure 1), at the Jackson Park site, were selected to be composited for a TIE study and chemical analysis of the pore water. No ordnance compounds were detected in the pore water. The TIE procedure indicated that both organic chemicals and trace metals were causative agents of the toxic effects in the fertilization tests, and ammonia could be contributing as well in the embryological development test. The overall toxicity, chemistry, and TIE test results indicate that ordnance compounds are not contaminants of concern at any of the sites studied in Puget Sound, Washington.

INTRODUCTION

Previous surveys have shown that sediments in the vicinity of Naval facilities in Puget Sound, Washington were contaminated with ordnance compounds, originating from past use, storage, improper disposal, and incineration of these compounds. It is not possible, however, to predict if sediment samples will be toxic on the basis of analytical chemistry information alone. Toxicity tests are recognized as effective tools to determine the biological significance of contamination found in coastal sediments.

There is a paucity of data in the scientific literature regarding the toxicity of ordnance compounds to marine organisms. A comprehensive literature search conducted by Oak Ridge National Laboratories (1996) on the available data on ordnance toxicity in sediments, resulted in little or no toxicity data for benthic marine or estuarine organisms. No Sediment Quality Standards (SQS) currently exist for these substances or their degradation products. The current study was undertaken, therefore, with the following primary objectives:

1) The generation of a marine toxicity database for the ordnance compounds of concern in the Pacific Northwest.

2) An assessment of the toxicity of sediments from Puget Sound suspected of contamination with ordnance compounds, followed by the application of Toxicity Identification Evaluation (TIE) studies with selected samples.

The marine toxicity database was generated for eight ordnance compounds: 2,4,6-trinitrotoluene (2,4,6-TNT), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), 1,3-dinitrobenzene (1,3-DNB), 1,3,5-trinitrobenzene (1,3,5-TNB), Royal Demolition Explosive (RDX), tetryl, and picric acid. Six toxicity test methods with five marine species and phyla, utilizing a total of nine different endpoints, were applied: fertilization and embryological development tests with the sea urchin *Arbacia punctulata*; zoospore germination test with the macro-alga *Ulva fasciata*; survival and reproduction test with the polychaete *Dinophilus gyrociliatus*; embryo-larval test with the redfish *Sciaenops ocellatus*; and survival test with juveniles of the opossum shrimp *Mysidopsis bahia*.

For the sediment assessment survey, surficial sediments were collected from 52 sites in Puget Sound. This included 25 stations in the vicinity of Jackson Park (Figure 1) and 25 in the vicinity of Port Hadlock (Figure 2) Naval Facilities, and 2 stations in Sequim Bay (SQ1 and SQ2), which was pre-selected as a reference site. Sediments were analyzed for porewater toxicity using the sea urchin, *A. punctulata*, fertilization and embryological development tests. The most toxic sediments were characterized chemically. Based on the combined results of the chemical analyses and toxicity tests, three stations adjacent to each other at the Jackson Park site, were selected for a combined TIE study. Sediment from the three sites was sampled and combined prior to the application of TIE procedures.

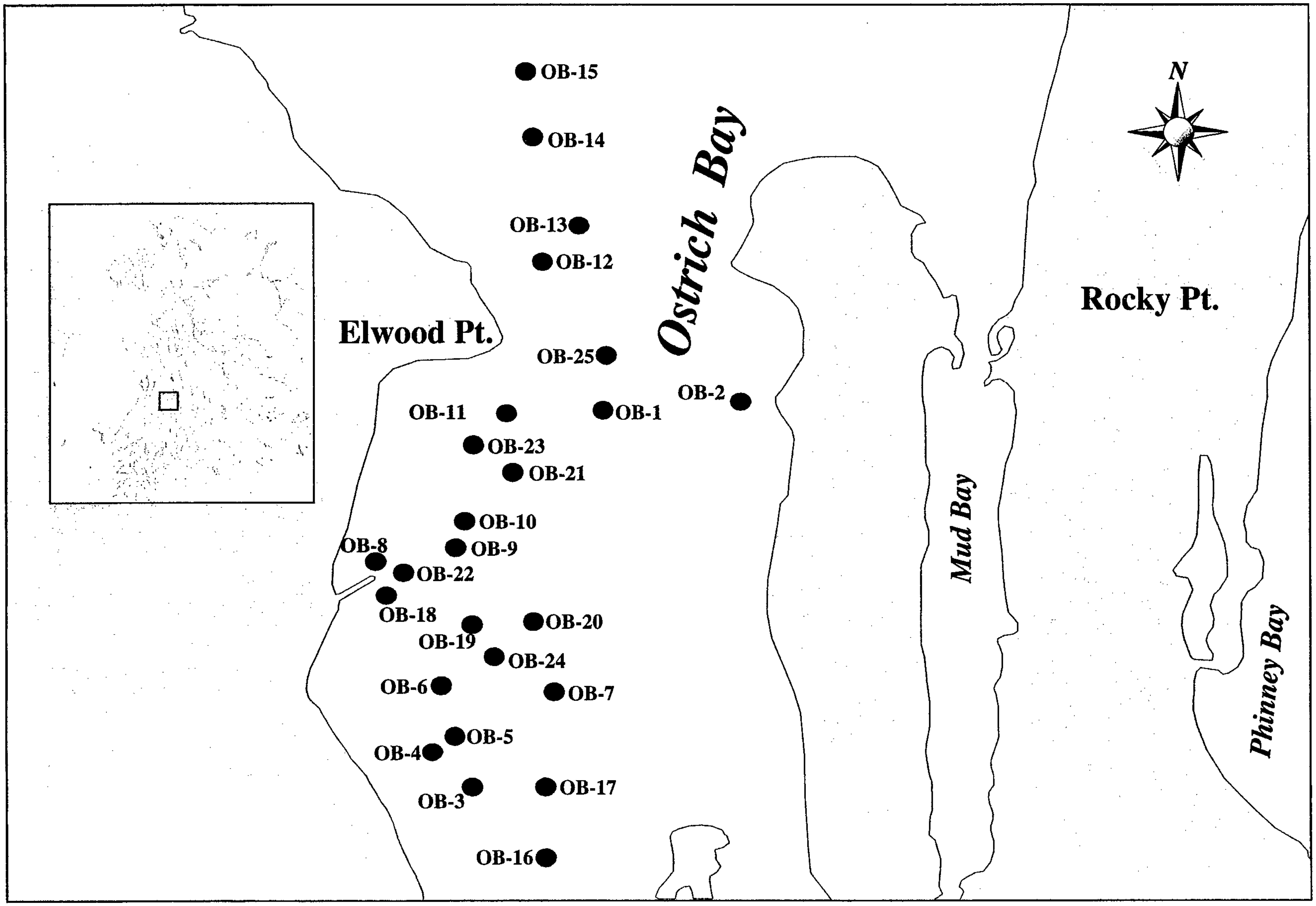

Figure 1. Sampling stations at Jackson Park site, Ostrich Bay, Puget Sound, Washington.

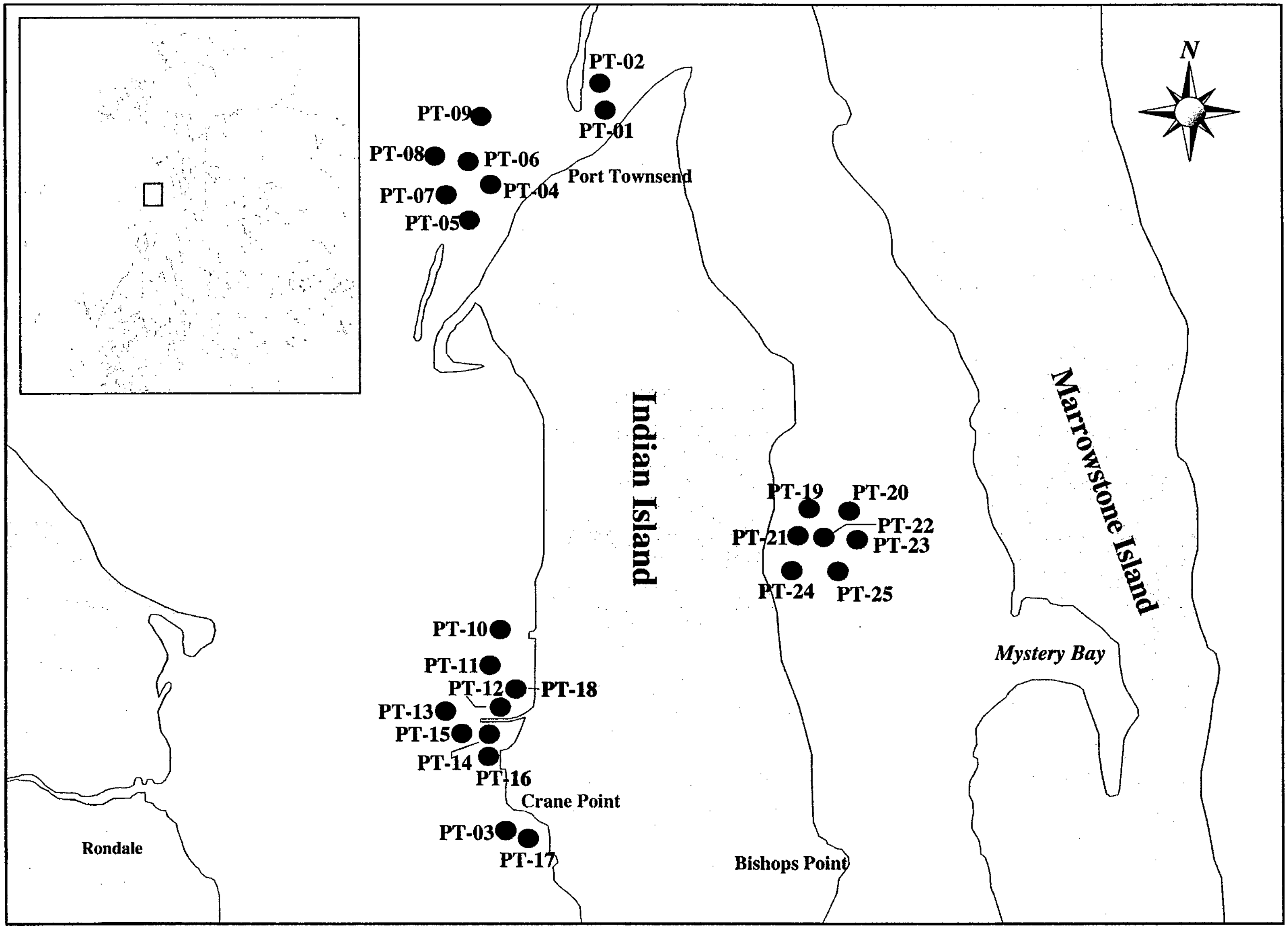

Figure 2. Sampling stations at Port Hadlock site, Puget Sound, Washington.

The specific objectives of this study were to:

1) Generate a marine toxicity database for eight ordnance compounds using six toxicity test methods with five marine species and phyla by:

- Spiking non-contaminated seawater with each of the ordnance compounds and analyzing acute and/or chronic effects with each of the selected standardized test methods.

- Calculating LC_{50} and/or EC_{50} (Lethal and Effective Concentration to 50% of the test organisms, respectively) values and No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) for each test method and endpoint.

2) Analyze the toxic effects of sediments from 50 stations from Puget Sound, Washington, and identify the most toxic stations by:

- Extracting pore water from surficial sediment samples and measuring the toxicity of three porewater dilutions (100, 50 and 25%) in one acute and one short-term chronic toxicity test using sea urchin gametes and embryos.

- Making statistical comparisons between test and reference stations for the sea urchin (*A. punctulata*) assays.

- Calculating EC_{50} values where possible.

3) Identify the chemicals responsible for adverse effects at the most toxic stations by:

- Applying TIE procedures to pore water from sediment from these stations.

MATERIALS AND METHODS

Phase I: *Generation of a Toxicity Database for Ordnance Compounds.*

Stock and Test Solution Preparation

Six of the chemicals used for the preparation of the stock and test solutions, picric acid, 2,4-DNT, 2,6-DNT, 2,4,6-TNT, 1,3-DNB and 1,3,5-TNB (S.G., ≥99% purity) were purchased from Chem Service (660 Tower Lane, West Chester, PA 19381-9941). Tetryl and RDX were not commercially available in the pure form, and were, therefore, re-crystallized and kindly donated by the Naval Surface Warfare Center, Indian Head Division, MD.

Stock solutions were prepared in standard dilution water (0.45 μm Millipore® filtered seawater - MFS) (see Attachment 1), with salinity adjusted to 30 ‰. The desired amount of the chemical, usually sufficient to generate a nominal concentration of 100 mg/L, but occasionally higher, was added to dilution water and vigorously stirred on a magnetic stirrer for 48 hours. RDX solutions were prepared on a heated stirrer and the solution was warmed up to 50 ± 5° C

for at least 30 hours to enhance dissolution in seawater. The remaining chemicals were usually stirred at room temperature. Each solution was filtered using a 0.45 μm Millipore® filter and kept at room temperature overnight, if prepared on the day before a test, or in the refrigerator, if kept for a longer period of time. The highest desired test solution of each chemical was prepared from the stock solution and serially diluted to 50% thereafter until all desired test concentrations were obtained.

Based on the results of range finding tests it was established that the use of a solvent carrier would be necessary to prepare some of the stock solutions for the sea urchin fertilization and embryological development tests, due to the low sensitivity of these methods to some of the chemicals. Stock solutions of 2,4-DNT, 2,6-DNT, 2,4,6-TNT, 1,3-DNB, 1,3,5-TNB and Tetryl were prepared by adding 2 ml of HPLC grade methanol and 100 mg of the chemical to a 20 ml scintillation vial and vigorously stirring on a magnetic stirrer for 2 hours. These solutions were then filtered though 0.45 μm Teflon® filters and added to 98 ml of dilution water. A solvent blank was also prepared with the highest concentration of methanol used in the test solutions of the sea urchin toxicity tests, along with the regular dilution water blank used as test control.

Chemical Analyses

Chemical concentrations of ordnance compounds in the test solutions and in test blanks (dilution water) were measured at test initiation and termination, following U.S. EPA Method 8330 (U.S.EPA, 1994). Analytes were measured against calibration curves prepared using the standards recommended in Method 8330. This method was modified for the measurement of picric acid, for which an isocratic mixture of 65% 0.1M sodium acetate buffer with pH adjusted to 4.8 and 35% methanol was used as mobile phase for the HPLC analysis. Picric acid standards in acetonitrile, at 1,000 μg/ml, were purchased from Chem Service and used for the method calibration.

Test Organisms

Arbacia punctulata urchins used in this study were obtained from Gulf Specimen Company, Inc. (Panacea, Florida). Polychaetes, *D. gyrociliatus*, have been in culture in our laboratory for over four years. Original organisms were isolated from material obtained from Long Beach Harbor, California. Fronds of the macro-alga *U. fasciata* were collected during an evening low tide on Port Aransas, Texas, jetties. Recently released eggs of the redfish, *S. ocellatus*, were kindly donated by the Texas Parks and Wildlife, GCCA-CPL Marine Development Center, Corpus Christi, Texas. Three-day old opossum shrimp, *M. bahia*, were purchased from Aquatic Indicators, Saint Augustin, Florida, and shipped overnight to our laboratory.

Toxicity Tests

All toxicity tests were conducted following standardized methodologies, although one modification was introduced to five of the six test methods: except for the sea urchin (*A. punctulata*) fertilization test, which has a one-hour exposure period, all tests were conducted in complete darkness. This modification was made to minimize photo-degradation of the ordnance compounds during the tests. The salinity of the test solutions was 30 ± 1 ‰, in all tests, and test

temperature was 20 ± 1° C, except for the redfish (*S. ocellatus*) test which was conducted at 25° C, to reproduce the temperature at which the eggs were released in the fish hatchery.

The sea urchin (*A. punctulata*) 30-minute fertilization and 48-hour embryological development tests, the polychaete (*D. gyrociliatus*) 7-day survival and reproduction test, and the alga (*U. fasciata*) 96-hour zoospore germination and germling growth test were conducted following SOPs F10.6, F10.7, F10.10 and F10.23, respectively (Attachments 2-5). The opossum shrimp (*M. bahia*) 96-hour survival test was conducted following the procedure established by (U.S. EPA, 1993), without test solution renewal during the exposure period. The redfish (*S. ocellatus*) 48-hour embryo hatching and larvae survival test was conducted based on the guidance of the ASTM standard method (ASTM, 1988).

Reference Toxicant Test

A reference toxicant test, using the surfactant sodium dodecyl sulfate (SDS), was conducted concurrently with each test series. For the sea urchin test, the EC_{50} values obtained in the reference toxicant tests were compared to a control chart prepared using the results of the 20 most recent tests conducted in our laboratory (Environment Canada, 1990). According to the control charts, the EC_{50} values for the fertilization test should fall between 2.9 and 7.6 mg/L, and for the embryological development test they should be between 1.8 and 6.8 mg/L. The SDS EC_{50} value obtained with the macro-algae, *U. fasciata*, test was compared to a control chart prepared with the results of 12 previous tests. Based on the control chart, the EC_{50} of a SDS test with *U. fasciata* zoospores should be between 1.2 and 5.7 mg/L. No control charts with SDS were available for the other organisms.

Water Quality Measurements

Initial water quality measurements, consisting of dissolved oxygen and pH, were made for each treatment of each test. Dissolved oxygen (DO) was measured with YSI® meters; pH was measured with an Orion® meters and pH probe. The salinity of the dilution water used to prepare stock and test solutions was adjusted to 30 ‰ prior to each test and was confirmed with a Reichert® or American Optical® refractometer. Water quality parameters were within acceptable limits for most samples. The pH of picric acid stock solutions was usually very low and was adjusted to 8.0 by addition of 1M NaOH.

Data Analysis

No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC), EC_{50} and/or LC_{50} (Effective and/or Lethal Concentration to 50% of the organisms) values were calculated for all tests using the concentrations of the tested chemicals measured at the beginning of each experiment. Prior to statistical analysis, the transformed data sets were screened for outliers (SAS, 1992). Outliers were detected by comparing the studentized residuals to a critical value from a *t*-distribution chosen using a Bonferroni-type adjustment. The adjustment is based on the number of observations, *n*, so that the overall probability of a type I error is at most 5%. The critical value, *cv*, is given by the following equation: $cv = t(\mathrm{df}_{\mathrm{Error}}, .05/(2 \times n))$.

After omitting outliers but prior to further analysis, the transformed data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992). Statistical comparisons among treatments for the assessment of NOEC and LOEC values were made using ANOVA and Dunnett's one-tailed *t*-test (which controls the experimentwise error rate) on the arcsine square root transformed data with the aid of SAS (SAS, 1989). The Trimmed Spearman-Karber method (Hamilton *et al.*, 1978) with Abbot's correction (Morgan, 1992) was used to calculate EC_{50} or LC_{50} values. For endpoints with continuous values, such as polychaete reproduction (number of laid eggs/adult female), and algae germling length and cell number, the test result was converted into percent of the control, and EC_{50} values were calculated using this percentage value.

A second criterion was also used with the two sea urchin tests to compare test means to reference means. Detectable significance criteria (DSC) were developed to determine the 95% confidence value based on power analysis of all similar tests performed by our lab (Carr and Biedenbach, 1999). This value is the percent minimum significant difference from the reference that is necessary to accurately detect a difference from the reference. The DSC value for the sea urchin fertilization assay is 15.5% at $\alpha \leq 0.05$, and 19% at $\alpha \leq 0.01$. For the embryological development test the DSC values at $\alpha \leq 0.05$ and $\alpha \leq 0.01$ are 16.4 and 20.6%, respectively.

Phase II: ***Porewater toxicity testing and TIE procedure.***

Sediment Sampling, Receipt and Tracking

Sampling sites in Puget Sound were selected based on Naval activities in the area and on previous information on ordnance contamination. Sampling was concentrated in two main areas: the Jackson Park site, with 25 stations selected in Ostrich Bay, (OB1-25, Figure 1); and the Port Hadlock site, with 25 stations selected adjacent to the Northern end of Indian Island, (PT1-25, Figure 2). Two pre-selected reference stations (SQ1 and SQ2) were located in Sequim Bay. Our laboratory reference station was located in Redfish Bay, Texas.

Sediment samples in Puget Sound were collected with the assistance of the Washington State Department of Ecology which provided the research vessel and sampling gear. Sampling was performed using a modified double Van Veen grab. Sediment samples were shipped to the U. S. Geological Survey (USGS) Marine Ecotoxicology Research Station (MERS) in Corpus Christi, Texas where the sediment was processed and tests were performed.

Surficial sediment samples were collected from the 52 selected stations in Puget Sound, in May 1998. Samples were placed in pre-cleaned one-gallon high-density polyethylene containers, chilled, and shipped in insulated coolers with blue ice. Separate sediment sub-samples were collected at stations OB1, OB8 and PT13, for replicate testing as part of the quality assurance program for the toxicity testing and chemical analyses. These were placed in separate containers and labeled OB27, OB26 and PT26, respectively. Samples were received by the USGS in Corpus Christi, Texas, the day following shipment. Shipments were accompanied by sample tracking sheets, and samples were logged into laboratory sample tracking systems. All porewater samples were extracted within three days from the time of field collection of sediment, and immediately upon arrival at the Corpus Christi laboratory.

Sediment Porewater Extraction Procedure

Pore water was extracted from the sediments using a pneumatic extraction apparatus, following SOP F10.9 (attachment 6). This extractor is made of polyvinyl chloride (PVC) and uses a 5 μm polyester filter. It is the same device used in previous sediment quality assessment surveys (Carr and Chapman, 1992, 1995; Carr *et al.*, 1996a, 1996b; NBS, 1993, 1994, 1995a, 1995b; USFWS, 1992; USGS, 1997a, 1997b, 1997c,1998, 1999; Carr and Nipper, 1998). After extraction, the porewater samples were centrifuged in polycarbonate bottles at 1200 x *g* for 20 min to remove any suspended particulate material; the supernatant was collected and frozen at -20° C. The pore water was stored frozen until just prior to testing, when water quality parameters were measured and adjusted, if necessary, following SOP 10.12 (Attachment 7).

Two days before conducting a toxicity test, the samples were moved from the freezer to a refrigerator at 4° C. One day prior to testing, samples were thawed in a tepid (20° C) water bath. Temperature of the samples was maintained at 20 ± 1° C. Sample salinity was measured and adjusted to 30 ± 1 °/₀₀, if necessary, using purified deionized water or concentrated brine at 122°/₀₀ salinity (see SOP F10.12, Attachment 7) with 10% reference porewater from Redfish Bay, Texas, added. Other water quality measurements (dissolved oxygen, pH, sulfide and ammonia concentrations) were made. Temperature and dissolved oxygen (DO) were measured with YSI® meters; salinity was measured with a Reichert® or American Optical® refractometer; and pH, sulfide (as S^{-2}), and total ammonia (expressed as nitrogen; NH_4) were measured with Orion® meters and their respective probes. Unionized ammonia concentrations (expressed as nitrogen; NH_3) were calculated for each sample using the respective salinity, temperature, pH, and NH_4 values. Following water quality measurements and adjustments, the samples were stored overnight at 4° C but returned to 20 ± 1° C before the start of the toxicity tests.

Toxicity Tests

Toxicity of the sediment pore water was determined using the sea urchin fertilization and embryological development tests with *A. punctulata*, following the procedures outlined in SOPs F10.6 and F10.7 (Attachments 2 and 3). *Arbacia punctulata* urchins were obtained from Gulf Specimen Company, Inc. (Panacea, Florida).

Each of the 55 porewater samples (including the three replicate sub-samples) was tested in a dilution series design at 100, 50, and 25% of the water quality adjusted sample with 5 replicates per treatment. Dilutions were made with 0.45 μm Millipore® filtered seawater (MFS). A reference porewater sample collected from Redfish Bay, Texas, which had been handled identically to the test samples, was included with each toxicity test as a negative control. This site is far removed from any known sources of contamination and has been used previously as a reference site (Carr and Chapman, 1992; NBS, 1993, 1994, 1995a, 1995b; USGS, 1997, 1998a, 1998b). In addition, dilution blanks of MFS and brine controls (purified deionized water with brine added to reach a 30 °/₀₀ salinity), were also included. The brine control had the objective of identifying any possible adverse effects caused by the brine. A dilution series test with sodium dodecyl sulfate (SDS) was included as a positive control and results were compared to the respective control charts.

Chemical Analyses

Thirteen sediment samples and the replicate sample of station OB8 (OB26) were selected for chemical analyses. The samples selected were from stations OB6, OB8, OB12, OB15, OB16, OB18, OB22 and OB23, from the Jackson Park site, and PT7, PT11, PT12, PT16 and PT19 from the Port Hadlock site. These sediments were shipped on dry ice to Columbia Analytical Services Inc., (C.A.S.), Kelso, Washington with chain of custody forms for analyses. Chemical analyses included a suite of ordnance compounds, trace metals, polycyclic aromatic hydrocarbons (PAHs), organochlorinated pesticides, polychlorinated biphenyls (PCBs), and butyltins, as well as particle size distribution. Ordnance compounds were measured by HPLC using Method 8330 (U.S. EPA SW846,1996), and trace metals were measured by ICP/MS using EPA Methods 200.8 (U.S EPA,1996) except for mercury which was measured by CVAA using EPA Method 7471 (U.S. EPA SW846, 1996). PAHs were measured by GC/MS using GC/MS selected ion monitoring as developed by C.A.S. PCBs were measured by EPA Method 8082 (U.S. EPA SW846,1996) using GC/ECD. Organochlorinated pesticides were measured using method 8081A (U.S. EPA SW846, 1996). Butyltins were measured by GC/FPD using the Columbia Analytical Protocol. Particle size distribution was analyzed by method PS-PSEP, modified (PSEP, 1986).

Data Analysis

The EC_{50} values for dilution series toxicity tests was calculated by the Trimmed Spearman-Karber method (Hamilton *et al.*, 1978) with Abbot's correction (Morgan, 1992). Statistical comparisons between each treatment and the reference pore water were made using ANOVA and Dunnett's one-tailed *t*-test (which controls the experimentwise error rate) on the arcsine square root transformed data with the aid of SAS (SAS, 1989). Prior to statistical analysis, the transformed data sets were screened for outliers (SAS, 1992). Outliers were detected by comparing the studentized residuals to a critical value from a *t*-distribution chosen using a Bonferroni-type adjustment. The adjustment is based on the number of observations, *n*, so that the overall probability of a type I error is at most 5%. The critical value, *cv*, is given by the following equation: $cv = t(df_{Error}, .05/(2 \times n))$. After omitting outliers but prior to further analysis, the transformed data sets were tested for normality and for homogeneity of variance using SAS/LAB® Software (SAS, 1992).

A second criterion was also used to compare test means to reference means. Detectable significance criteria (DSC) were developed to determine the 95% confidence value based on power analysis of all similar tests performed by our lab (Carr and Biedenbach, 1999). This value is the percent minimum significant difference from the reference that is necessary to accurately detect a difference from the reference. The DSC value for the sea urchin fertilization assay is 15.5% at $\alpha \leq 0.05$, and 19% at $\alpha \leq 0.01$. For the embryological development test the DSC values at $\alpha \leq 0.05$ and $\alpha \leq 0.01$ are 16.4 and 20.6%, respectively.

Toxicity Identification Evaluation (TIE) Procedures - Phase 1

Based on the results of the toxicity tests and of chemical analyses, three of the most toxic stations, which were located in Ostrich Bay and adjacent to each other, were selected for the TIE procedure. Seven gallons of sediment from each station were collected in August 1998,

composited, and shipped to the U. S. Geological Survey (USGS) Marine Ecotoxicology Research Station (MERS) in Corpus Christi, Texas. Pore water was extracted from this sediment composite upon arrival to the laboratory, and processed following the protocol described in Attachment 6.

The sea urchin fertilization and embryological development tests were used with the TIE procedure. Initially, the toxicity of a frozen and a fresh porewater sample was compared. Since no significant difference in toxicity was detected between the samples, frozen pore water was used for the TIE procedure and treated as described in the previous sections.

Baseline toxicity of the sample was assessed. Phase I TIE treatments were applied following the USEPA protocol (Burgess *et al.*, 1996) and consisted of:

- Aeration, for the assessment of the contribution of volatile chemicals to the toxicity;
- Filtration, for the assessment of the contribution of particulate material to the toxicity;
- C_{18} column, for the assessment of the contribution of organic chemicals to the toxicity;
- EDTA addition, for the assessment of the contribution of metals to the toxicity;
- Sodium thiosulfate addition, for the assessment of the contribution of oxidants to the toxicity;
- pH increase and reduction, for the assessment of the contribution of ammonia to the toxicity.

Chemical Analyses

A sub-sample (4 L) of the frozen porewater samples used for the TIE procedure was shipped to on dry ice to Columbia Analytical Services, Inc., Kelso, Washington, with chain of custody forms for chemical analyses. A sub-sample (4 L) of the fresh porewater sample used for the initial comparison of fresh and frozen porewater toxicity was also sent for chemical analyses. The same methods described for the sediment analyses were applied for chemical measurements conducted with the pore water.

Data Analysis

Statistical comparisons between each TIE treatment applied to the porewater sample from Ostrich Bay and the baseline toxicity of that same sample were made using ANOVA and Dunnett's two-tailed *t*-test on the arcsine square root transformed data with the aid of SAS (SAS, 1989).

RESULTS AND DISCUSSION

PHASE I: ***Generation of a toxicity database for ordnance compounds.***

Toxicity Tests

Toxicity differed considerably both among the eight tested ordnance compounds and among test methods and endpoints. The EC/LC_{50}, NOEC and LOEC values for the different chemicals and endpoints ranged from <0.02 to 415 mg/L (Tables 1 and 2). A complete set of the raw data for all the toxicity tests with ordnance compounds is presented in Appendix A.

The polychaete, *D. gyrociliatus*, test had multiple endpoints. The reproductive endpoint, represented by the number of eggs laid per adult female, was consistently more sensitive than the survival endpoint. The macro-algae zoospore test with *U. fasciata* also had multiple endpoints, and germling length was the most sensitive, oftentimes matched by germling cell number. Percent germination was the least sensitive of the three endpoints. Graphic illustrations of the results for the most sensitive endpoint of each kind of toxicity test, by chemical, are presented in figures 3-10.

The macro-algae zoospore germination test, and particularly the germling length endpoint, was the most sensitive overall. However, it was not as sensitive as the polychaete reproduction endpoint with 2,6-DNT, the mysid survival endpoint with picric acid, and the polychaete reproduction and urchin embryological development endpoints with tetryl. The polychaete reproduction endpoint was the most sensitive with 2,6-DNT and tetryl, and the second most sensitive with 1,3-DNB, 1,3,5-TNB and RDX. Mysid survival was the most sensitive endpoint with picric acid, and tended to be the second or third most sensitive endpoint with the other compounds. The macro-algae germination and germling length and cell number, and the polychaete reproduction endpoints were the only ones for which an EC_{50} could be calculated for RDX. All the other test organisms and endpoints were not significantly affected by this chemical at or below its solubility limit.

In summary, macro-algae (*U. fasciata*) zoospore germination, polychaete (*D. gyrociliatus*) reproduction, and mysid (*M. bahia*) survival tended to be more strongly affected by ordnance compounds than were redfish (*S. ocellatus*) larvae survival and sea urchin (*A. punctulata*) fertilization and embryological development. Sea urchin fertilization was the least sensitive test endpoint overall, while urchin embryological development exhibited relatively high sensitivity to 2,6-DNT, 2,4,6-TNT, 1,3-DNB, 1,3,5-TNB and tetryl.

Picric acid was very soluble, but presented low toxicity, whereas its parent compound, tetryl, was the most toxic chemical tested overall. Tetryl was one to three orders of magnitude more toxic than picric acid (note different scales in Figures 9 and 10). The second most toxic ordnance compound overall was 1,3,5-TNB, although it was in the same range of toxicity of 2,4,6-TNT for mysid survival. The EC_{50} and LOEC values of 1,3,5-TNB were usually one order of magnitude lower than those of 1,3-DNB, indicating higher toxicity of the former (Tables 1 and 2). The only marine toxicity data found in the literature for 1,3,5-TNB, tetryl, or picric acid were for the marine bacteria bioluminescence test with *Vibrio fischeri* (Microtox®), which tends to be used mostly as a screening test, and for the periwinkle *Littorina littorea*. This data is in agreement with that from the present study: the EC_{50} of 1,3,5-TNB in the Microtox® test was similar to that of tetryl, and three orders of magnitude higher than the EC_{50} for its degradation product, picric

Table 1. EC_{50} and LC_{50} (mg/L) data for toxicity tests conducted with ordnance compounds. Ninety-five percent confidence intervals in parenthesis.

		EC_{50}/LC_{50} (mg/L)							
Organism	Endpoint	2,4-DNT	2,6-DNT	2,4,6-TNT	1,3-DNB	1,3,5-TNB	RDX	Tetryl	Picric Acid
Sea urchin	Fertilization	68 (61-76)	>84	>103	258 (217-309)	84 (76-93)	>75	3.0 (2.5-3.5)	349 (321-380)
	Embryo development	51.4 (49-54)	6.7 (6.1-7.3)	12 (11-12.5)	92 (NR)[a]	1.3 (1.2-1.4)	>75	0.08 (0.07-0.08)	281 (267-296)
Algae	Germination	2.5 (2.4-2.6)	6.7 (6.2-7.3)	2.5 (2.47-2.61)	0.85 (0.8-0.9)	0.08 (0.07-0.09)	12 (11.5-12.4)	0.67 (0.65-0.70)	415 (389-443)
	Germling length	1.7 (1.5-2.0)	2.9 (2.3-3.6)	0.76 (0.44-1.30)	0.41 (0.36-0.47)	0.05 (0.05-0.06)	8.1 (7.1-9.4)	0.34 (031-0.39)	94 (74-120)
	Germling cell number	2.1 (1.8-2.5)	4.2 (3.5-5.2)	1.4 (1.0-1.9)	0.45 (0.38-0.54)	0.06 (0.05-0.07)	9.8 (8.8-11.0)	0.40 (0.35-0.45)	118 (101-138)
Polychaete	Survival	21 (19-23)	13 (12-15)	7.7 (7.3-8.1)	15 (14-16)	2.1 (1.7-2.0)	>49	0.06 (0.05-0.07)	265 (NR)*
	Laid eggs/female	5.7 (4.9-6.4)	2.1 (1.4-3.1)	1.8 (1.6-2.0)	3.7 (3.4-4.1)	0.60 (0.54-0.68)	26 (13-52)	0.02 (0.02-0.03)	155 (149-160)
Redfish	Larval survival	48 (NR)	34 (26-44)	8.2 (NR)[a]	46 (35-60)	1.4 (NR)[a]	>68	1.8 (NR*)	127 (113-143)
Mysid	Survival	5.4 (4.2-6.9)	5.6 (4.4-7.0)	0.98 (0.73-1.32)	7.1 (NR)[a]	1.3 (1.0-1.6)	>47	1.3 (1.0-1.5)	13 (11-16)

[a] 95% confidence limits not reliable; usually occurs when there is 0% effect in one concentration and 100% effect in the next, generating a 2-point curve, which does not allow for the calculation of confidence limits.

Table 2. NOEC and LOEC data for toxicity tests conducted with ordnance compounds.

		NOEC/LOEC (mg/L)							
Organism	**Endpoint**	2,4-DNT	2,6-DNT	2,4,6-TNT	1,3-DNB	1,3,5-TNB	RDX	Tetryl	Picric Acid
Sea urchin	Fertilization	39 / 75	23 / 45	103 / >103	84 / 110	35 / 48	75 / >75	<0.6 / 0.6	178 / 352
	Embryo development	18 / 39	<5.0 / 5.0	2.1 / 9.1	<84 / 84	0.24 / 0.48	75 / >75	0.036 / 0.083	178 / 352
Algae	Germination	0.94 / 1.8	2.2 / 4.7	1.7 / 3.4	0.30 / 0.65	0.046 / 0.093	9.2 / 15.7	0.50 / 1.0	169 / 336
	Germling length and cell number[a]	<0.48 / 0.48	<1.2 / 1.2	<0.21 / 0.21	<0.21 / 0.21	0.029 / 0.046	<5.0 / 5.0	0.098 / 0.25	<92 / 92
Polychaete	Survival	9.5 / 19.0	14.6 / 29.6	6.1 / 11.6	9.7 / 19.6	1.2 / 2.4	49 / >49	0.026 / 0.056	199 / 379
	Laid eggs/ female	<2.4 / 2.4	<1.8 / 1.8	1.4 / 2.8	2.4 / 4.4	0.35 / 0.61	11.9 / 23.7	0.015 / 0.026	108 / 198
Redfish	Larval survival	34.6 / 66.8	13.7 / 32.0	6.3 / 10.8	25.2 / 49.6	0.99 / 2.00	68 / >68	1.2 / 2.6	97 / 187
Mysid	Survival	3.6 / 6.8	5.0 / 9.8	0.65 / 1.34	5.2 / 9.7	0.96 / 1.88	47 / >47	1.1 / 2.0	9.2 / 20.6

[a] NOEC and LOEC values were the same for germling length and cell number, and therefore, data for the two endpoints were combined.

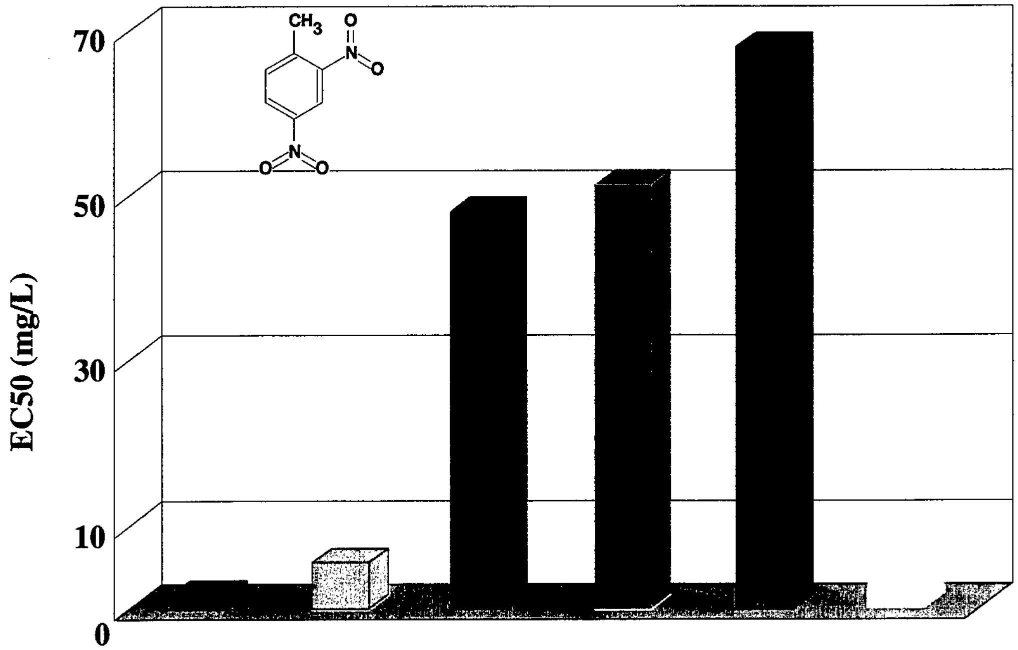

Figure 3: EC$_{50}$ values of 2,4-dinitrotoluene for the most sensitive endpoint of six different toxicity tests with five marine species.

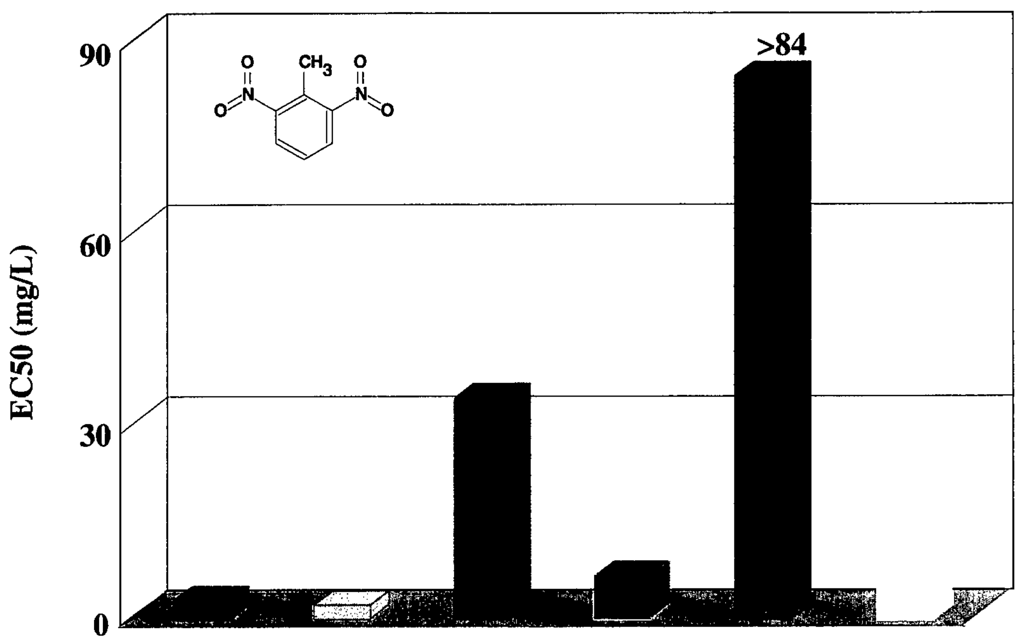

Figure 4: EC$_{50}$ values of 2,6-dinitrotoluene for the most sensitive endpoint of six different toxicity tests with five marine species.

Algae germling length; Polychaete eggs/adult; Red fish larvae survival;

Sea urchin embryo development; Sea urchin fertilization; Mysid survival

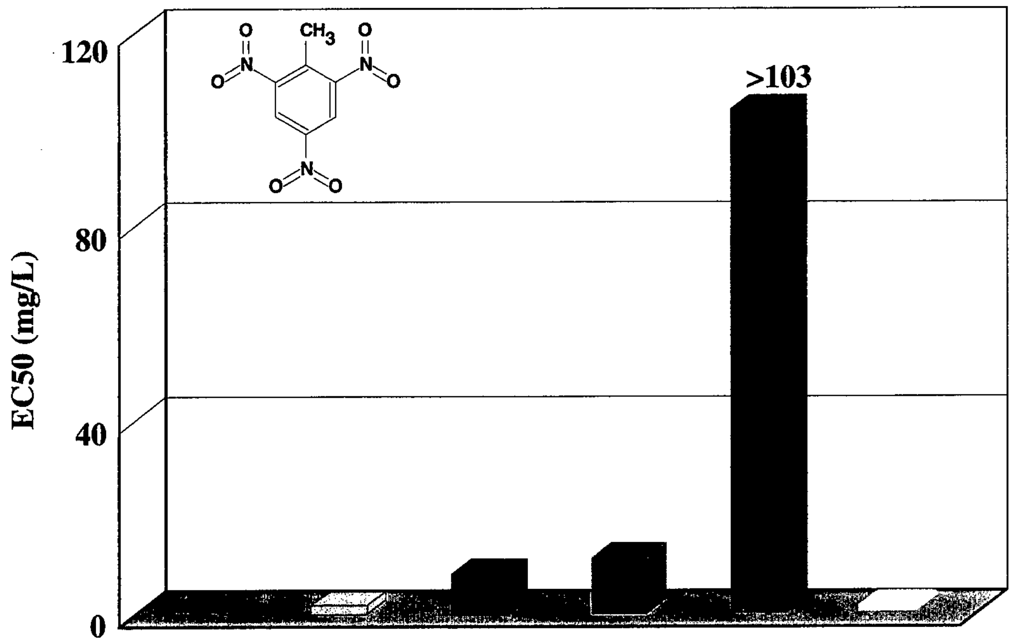

Figure 5: EC_{50} values of 2,4,6-trinitrotoluene for the most sensitive endpoint of six different toxicity tests with five marine species.

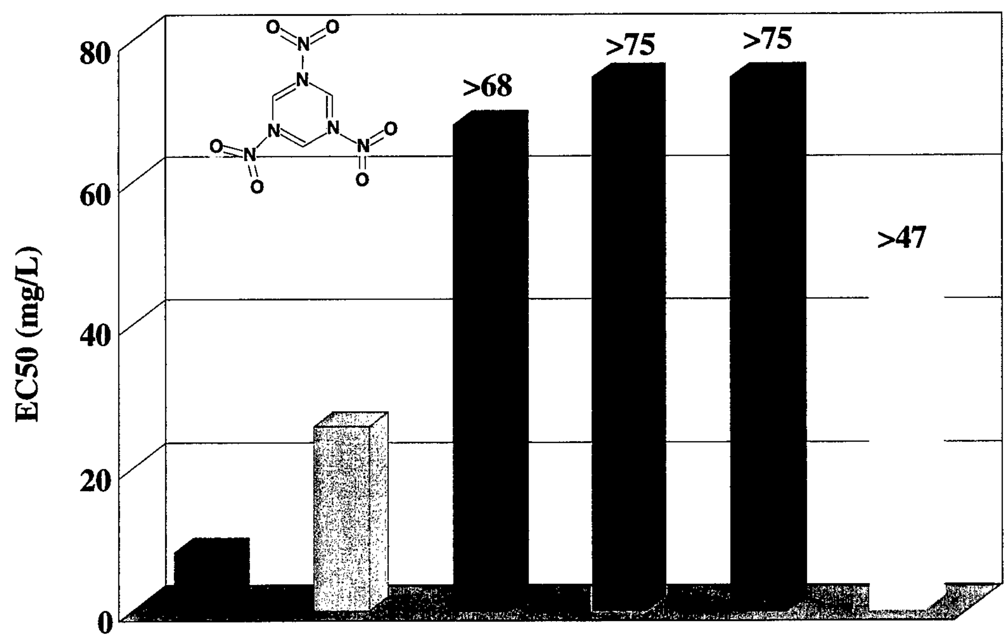

Figure 6: EC_{50} values of RDX for the most sensitive endpoint of six different toxicity tests with five marine species.

Algae germling length; Polychaete eggs/adult; Red fish larvae survival;

Sea urchin embryo development; Sea urchin fertilization; Mysid survival

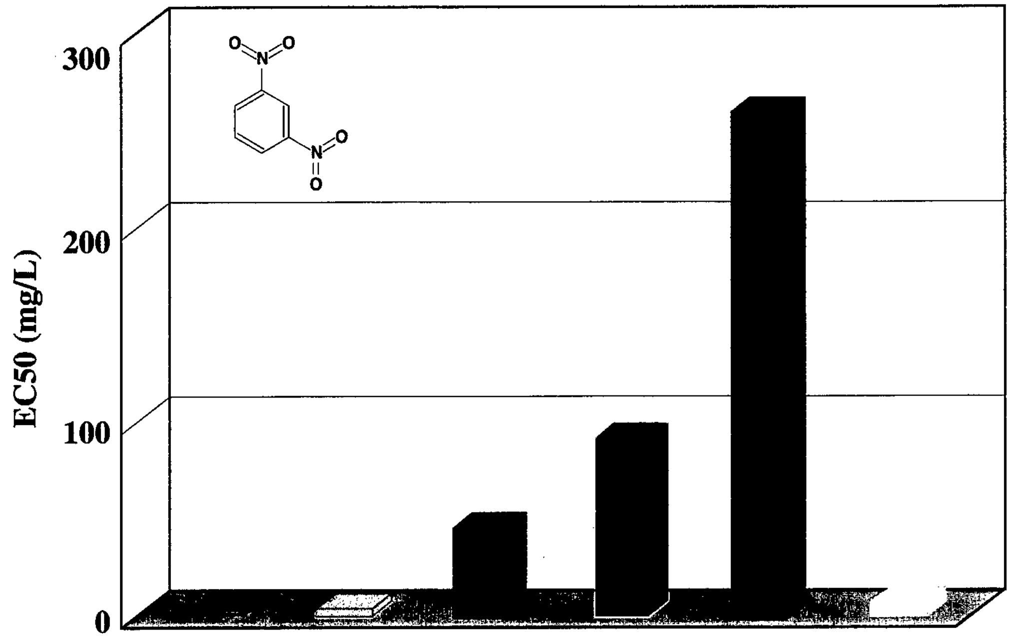

Figure 7: EC_{50} values of 1,3-dinitrobenzene for the most sensitive endpoint of six different toxicity tests with five marine species.

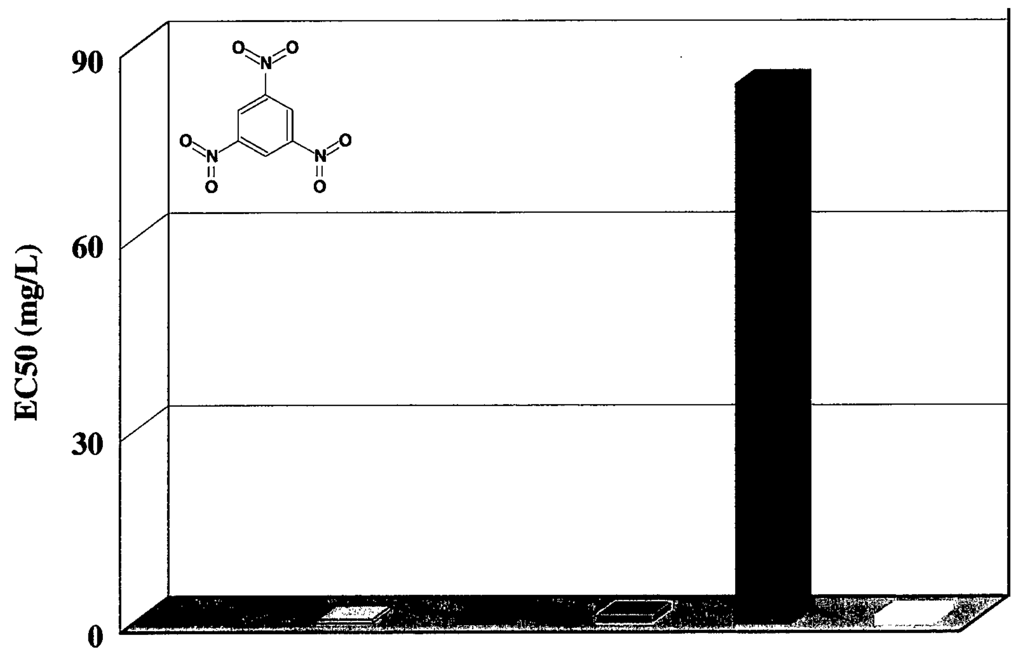

Figure 8: EC_{50} values of 1,3,5-trinitrobenzene for the most sensitive endpoint of six different toxicity tests with five marine species.

Algae germling length; Polychaete eggs/adult; Red fish larvae survival;

Sea urchin embryo development; Sea urchin fertilization; Mysid survival

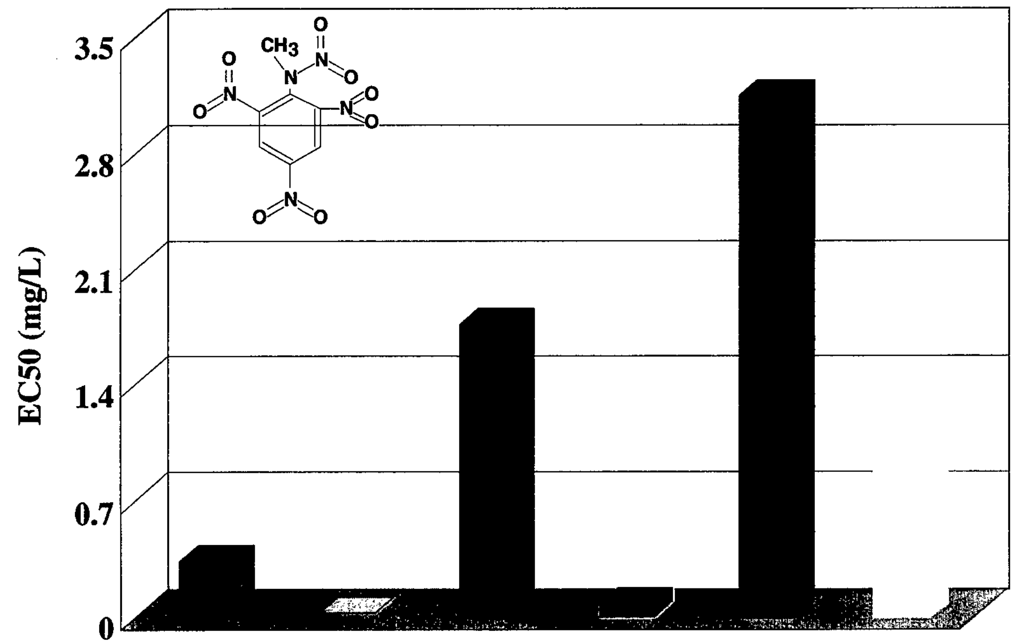

Figure 9: EC$_{50}$ values of Tetryl for the most sensitive endpoint of six different toxicity tests with five marine species.

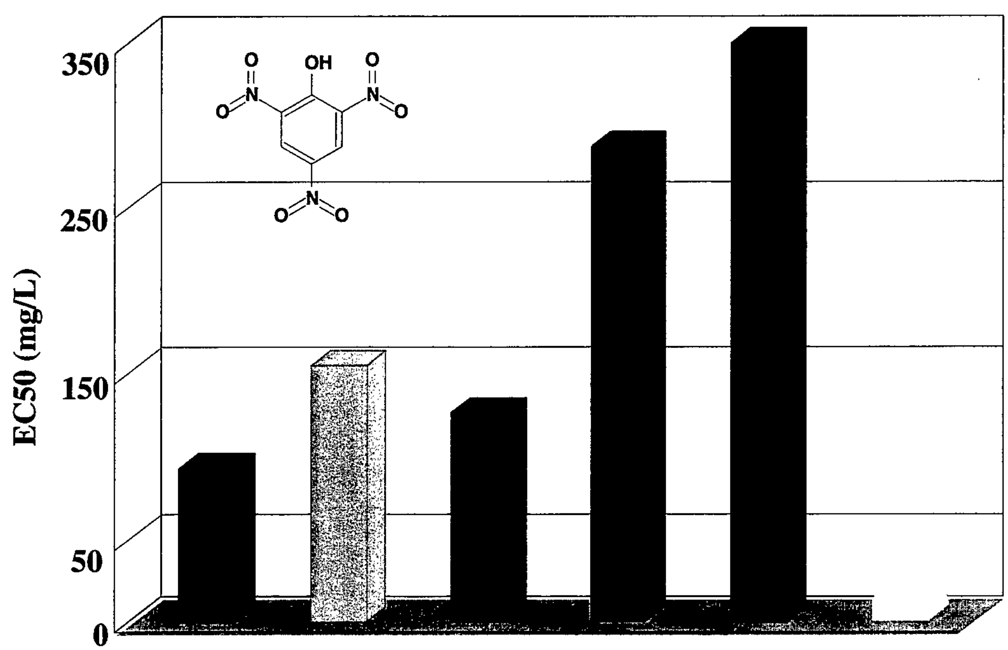

Figure 10: EC$_{50}$ values of Picric Acid for the most sensitive endpoint of six different toxicity tests with five marine species.

Algae germling length; Polychaete eggs/adult; Red fish larvae survival;

Sea urchin embryo development; Sea urchin fertilization; Mysid survival

acid (Drzyzga *et al.*, 1995). Picric acid also exhibited low toxicity to the periwinkle *L. littorea*, with 48- and 96-hr LC_{50} values of 336 and 57 mg/l, respectively (Carajaville *et al.*, 1989).

The solubility of RDX in seawater is reported in the literature as 56 mg/l, at approximately 25° C (Hoffsomer and Rosen, 1973). We occasionally achieved slightly higher concentrations of RDX, but toxicity was not observed for five of the nine test endpoints. However, RDX was toxic to all the algae test endpoints (EC_{50} between 8 and 12 mg/l), as well as for the reproduction endpoint of the polychaete test (Tables 1 and 2). Drzyzga *et al.* (1995) reported a 30-minute;EC_{50} of 75 mg/L for RDX in the Microtox® test.

In the sea urchin embryological development test the EC_{50} for 2,6-DNT was one order of magnitude lower than for 2,4-DNT. The toxicity of the 2,6-DNT was similar to that of 2,4,6-TNT (Figures 4 and 5). The only other data set with sea urchin embryos found in the literature was for *Lytechinus variegatus*. With this species the two DNT isomers caused the development of abnormal pluteus larvae at a 10 mg/L concentration. Only a slight retardment of the embryological development was observed at 10 mg/l of 2,4,6-TNT (Davenport *et al.*, 1994), whereas in the present study 50% of the embryos were strongly retarded or abnormal at a 12 mg/L concentration (Table 1). The two DNT isomers were similarly toxic in the other 5 tests with 8 different endpoints. Slightly higher EC_{50} values for 2,4,6-TNT than for 2,4- and 2,6-DNT show that the former tended to be slightly more toxic than the DNTs (Tables 1 and 2). Data from the literature indicates that 2,4-DNT tends to be less or equally toxic than 2,6-DNT in the Microtox® test, with 2,4,6-TNT more toxic than 2,6-DNT (Deneer *et al.*, 1989; Johson *et al.*, 1994; Drzyzga *et al.*, 1995; Dodard *et al.*, in press). The 96- and 72-hour LC_{50} values of 2,4,6-TNT to the marine copepod, *Tigriopus californicus*, and larvae of the oyster *Crassostrea gigas*, calculated with data presented in the literature (Won *et al.*, 1976), were 5.3 and 8.2 mg/l, respectively. This is in the same range of the results of similar tests (opossum shrimp survival and sea urchin embryological development) conducted in the present study. The increase of toxicity to marine organisms, from dinitro- to trinitrotoluene and from dinitro- to trinitrobenzene, observed both in the present study and in the literature data, suggests that the level of toxicity of ordnance compounds tends to increase with the number of nitrogen radicals.

Reference Toxicant Test

Reference toxicant tests with SDS conducted concurrently with each toxicity test indicated similar sensitivity of most of the test methods and endpoints (Appendix A10). The EC_{50} values ranged from 1.3 to 7.1 mg/L, except for the mysid survival test, which was less sensitive to SDS, with an EC_{50} 16.5 mg/L. The SDS EC_{50} values for the sea urchin fertilization and embryological development tests were within the acceptable range, according to our laboratory's updated control charts. The SDS EC_{50} value for the macro-algae zoospore germination endpoint was also within the range obtained in previous tests, indicating that the zoospores used in the test with ordnance compounds were within the normal range of sensitivity. No control charts were available for the SDS tests with the polychaete, fish embryos and opossum shrimp.

Water Quality Measurements

Water quality in the test solutions was generally within the expected range for each kind of test. Test salinity was always 30 ± 1 ‰, and was not measured for all test solutions, since these

were prepared with dilution water with salinity previously adjusted to the desired value. Water quality measurements for the toxicity tests are presented in Appendix B. Dissolved oxygen in the test solutions ranged from 84 to 121% saturation, except for the opossum shrimp test termination, where it was lower than 80% but higher than 70% saturation in a few test jars, probably as a result of the accumulation of brine shrimp nauplii used to feed the test organisms (Appendix B6). This does not seem to have affected survival, which was high at the non-toxic concentrations of the ordnance compounds in spite of the relatively low D.O. (Appendix A9).

Ammonia was not measured on a regular basis in the test solutions, since it was measured in the dilution water used to prepare them, and was always very low or nil. However, ammonia was measured at mysid test termination, since a build up of this product as a result of the brine shrimp accumulation could be a factor of concern. Unionized ammonia (NH_3) levels in mysid test jars after the 96-hour exposure ranged from 0.3 to 13.7 μg/L (Appendix B6). These levels well below the NOEC for this species and were not responsible for the toxicity observed in the test.

The initial pH of all test solutions was 8.0 ± 0.2 (Appendix B). The pH at test termination was also within this range for the redfish and polychaete tests. In the opossum shrimp test the final pH tended to be slightly lower (7.5 ± 0.2), probably as a result of respiratory activity of the excess brine shrimp inserted as food, raising the level of CO_2 in the water and therefore increasing acidity. Picric acid stock solutions had very low pH, which was adjusted to 8.0 prior to test initiation. The pH in picric acid test solutions tended to decrease during the exposure period but never dropped below 7.0. Water quality was not measured at sea urchin and algae test termination due to the need of immediate preservation of the samples with formalin for future analysis.

Chemical Analysis

The concentrations of ordnance compounds in all test solutions were measured at test start and end, except for the sea urchin fertilization test, which has a one-hour exposure time. For this test, concentrations were only measured at test start, since no loss by degradation was expected in such a short term. Concentrations for the sea urchin fertilization test are presented in Appendix A1, along with the toxicity test data. The measured concentrations of ordnance compounds in all test solutions at test initiation and termination for all experiments except for the sea urchin fertilization test, are presented on Appendix C. The percentage of the initial concentration still remaining at test termination is also presented, as an indication of loss of the chemical during the exposure period. In some cases the final amount of some chemicals is indicated as higher than the initial (% of initial >100). This is likely to be a measurement error, related to the intrinsic variability of the chemical method applied. In the macro-alga, *U. fasciata*, the measured concentration of RDX at test termination was up to 30% higher than at test initiation in the three highest concentrations (Appendix C2). This may have been due to the fact that the samples from test initiation were frozen prior to the chemical analyses, and part of the RDX, which was close to its solubility limit, could have re-crystallized. The chemical extraction method with methanol may not have been sufficient to completely re-dissolve the RDX. While some of the ordnance compounds exhibited little or no loss during the test exposure periods, others seemed to degrade significantly. Tetryl and 1,3,5-TNB exhibited the highest losses during most tests. In most cases the rate of loss of the chemicals from the water column was in inverse proportion to the initial

concentration, with higher percentage of loss in the solutions with lower initial concentrations, with gradually smaller percent loss towards the higher concentrations.

Comparing the two 48-hour tests with sea urchin and redfish (Appendices C1 and C4), conducted at 20 and 25° C, respectively, a loss of 1,3,5-TNB only occurred in the fish test, with final concentrations ranging from 13 to 81% of the initial. Tetryl concentrations decreased form test start to end in both methods, with final concentrations in the urchin test ranging from 0 to 85% of the initial, whereas in the fish test they were 27 ± 2% of the initial for all tested concentrations. Up to 60% of the 2,4,6-TNT was also lost in some concentrations of the fish test. Higher degradation rates in this test were expected due to the higher temperature which would enhance bacterial activity and, therefore, biodegradation. All other tests were conducted at 20° C, and therefore temperature variations were not expected to be a contributing factor in the variability of degradation rates among tests.

The two tests with a 96-hour exposure period, with macro-algae zoospores and juvenile mysids, had high losses of 1,3,5-TNB and tetryl, ranging from 0 to 30% of the initial concentration for the first, and 0 to 34% for the latter (Appendices C2 and C5). The concentrations of 2,4,6-TNT at test termination were also somewhat lower in the macro-alga test, but were reduced to nearly zero in the mysid test. Part of this loss may be due to degradation by enhanced bacterial activity caused by the accumulation of dead brine shrimp in the test jars. In order to assess if the loss of ordnance compound from test start to end could be due to adsorption to test organisms rather than to bio- and photo-degradation, one extra-vial of a selected concentration of each chemical was left without organisms in the macro-alga zoospore test. These extra-vials were submitted to the same test conditions as the other test vials, and chemical concentrations were measured at test termination. The final concentrations of all chemicals in the vials without organisms were in the same range of those in the vials with organisms added, indicating that the observed losses would have been due to degradation, rather than adsorption.

The polychaete test had the longest exposure period, of 7 days, and therefore was expected to show the highest degradation rates of ordnance compound during the test. Tetryl concentrations were reduced to zero (below detection) in all test concentrations, and 1,3,5-TNB and 2,4,6-TNT ranged from 0 to 80% of the initial concentration at test end (Appendix C3). Overall, the compounds with three nitro groups (TNT and TNB) exhibited higher degradation than those with two nitro groups (DNB and DNTs), and tetryl with its four nitro groups was also highly degradable in relatively short time periods of up to 7 days.

PHASE II: ***Porewater Toxicity Testing and TIE Procedure.***

Sea Urchin Fertilization Toxicity Test

Ostrich Bay, at the Jackson Park site, had more stations that were toxic in the urchin fertilization test than did the Port Hadlock site (Figures 11 and 12, Table 3, Appendix D1). Samples were considered toxic when their effect was significantly higher ($\alpha \leq 0.05$) than that of the reference sample and the percent fertilization was below the detectable significance criteria (DSC) at $\alpha \leq 0.05$ (Carr and Biedenbach, 1999). The most toxic samples were from stations OB12, OB16 and OB23, with significant toxicity at a 25% dilution. The samples from stations OB8, OB11, OB15, OB17 and OB18 were significantly toxic at a 50% dilution. In the 100%

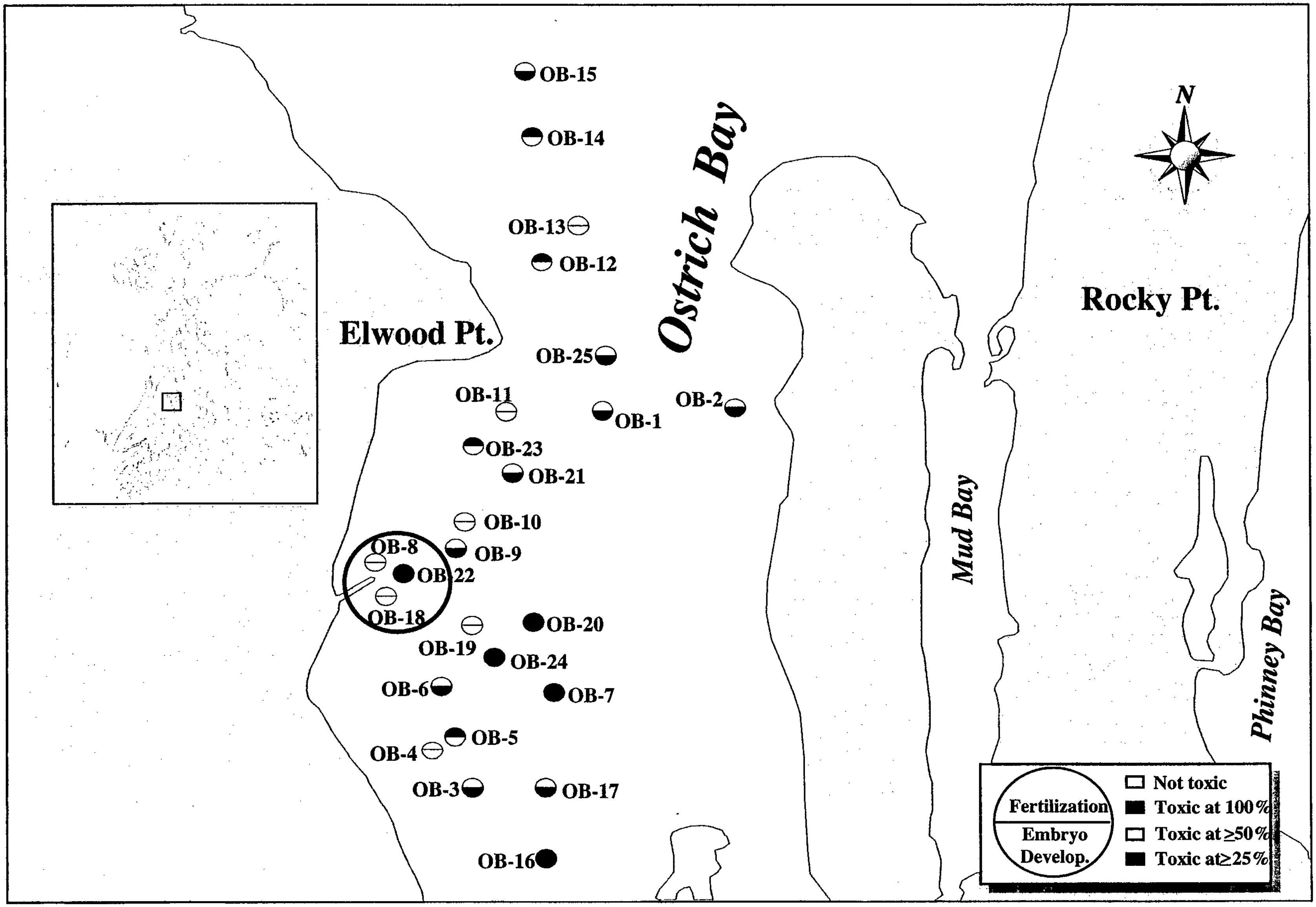

Figure 11. Sampling stations at Jackson Park site, Ostrich Bay, Puget Sound, Washington. Color differentiation of symbol indicates those stations that were significantly toxic relative to the reference site in the sea urchin (*A. punctulata*) fertilization and embryological development test (Dunnett's *t*-test, $\alpha \leq 0.05$ and detectable significance criteria applied).

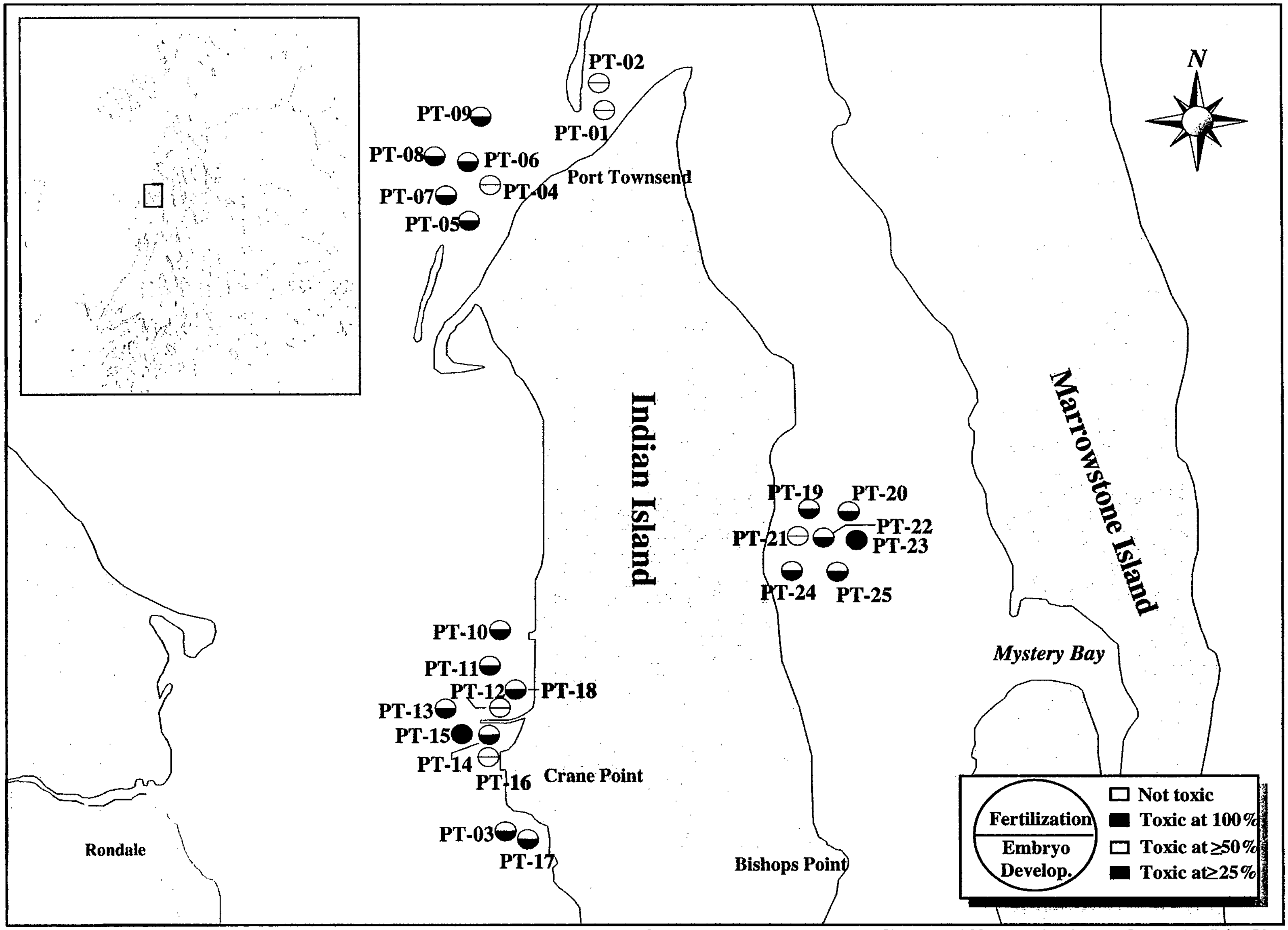

Figure 12. Sampling stations at Port Hadlock site, Puget Sound, Washington. Color differentiation of symbol indicates those stations that were significantly toxic relative to the reference site in the sea urchin (*A. punctulata*) fertilization and embryological development test (Dunnett's *t*-test, $\alpha \leq 0.05$ and detectable significance criteria applied).

Table 3. EC_{50} **toxicity values and statistical significance of sediment porewater samples from Puget Sound, Washington, assayed in the sea urchin fertilization and embryological development tests.**

Station	Fertilization Test			Embryological Development Test		
	EC_{50}[a]	95% Confidence Limits	Sign. Toxic Dilution[b]	EC_{50}[a]	95% Confidence Limits	Sign. Toxic Dilution[b]
OB1	>100	-	-	71.3	69.8-72.8	100
OB2	>100	-	-	68.4	65.5-71.4	100
OB3	>100	-	-	64.8	61.3-68.5	100
OB4	>100	-	-	33.7	NR[c]	100, 50
OB5	94.4	82.7-107.7	100	39.9	35.7-44.5	100, 50
OB6	>100	-	-	59.7	53.1-67.2	100, 50, 25
OB7	>100	-	100	66.0	63.3-68.8	100
OB8	54.4	50.0-59.1	100, 50	35.2	34.7-35.8	100, 50
OB9	>100	-	-	67.9	65.8-70.0	100
OB10	>100	-	-	42.1	39.4-45.0	100, 50
OB11	71.5	67.4-75.8	100, 50	33.1	NR[c]	100, 50
OB12	<25	-	100, 50, 25	32.2	NR[c]	100, 50
OB13	>100	-	-	56.8	52.0-62.1	100, 50
OB14	>100	-	100	34.4	NR[c]	100, 50
OB15	69.5	62.8-77.0	100, 50	31.3	NR[c]	100, 50, 25
OB16	40.6	35.4-46.5	100, 50, 25	65.5	60.3-71.3	100
OB17	88.6	76.8-102.2	100, 50	70.0	64.6-75.8	100
OB18	45.1	40.9-49.8	100, 50	34.0	32.9-35.0	100, 50
OB19	>100	-	-	56.8	52.3-61.8	100, 50
OB20	83.9	77.5-90.7	100	64.5	59.9-69.4	100
OB21	>100	-	-	>100	-	100
OB22	>100	-	100	28.6	NR[c]	100, 50, 25
OB23	50.8	35.3-73.0	100, 50, 25	44.1	39.2-49.5	100, 50
OB24	94.5	83.0-107.6	100	65.5	61.5-69.8	100
OB25	>100	-	-	83.9	78.0-90.2	100

Table 3: Continued

Station	EC_{50}[a]	95% Confidence Limits	Sign. Toxic Dilution[b]	EC_{50}[a]	95% Confidence Limits	Sign. Toxic Dilution[b]
PT1	>100	-	-	>100	-	-
PT2	>100	-	-	>100	-	-
PT3	>100	-	-	90.8	76.9-107.2	100
PT4	>100	-	-	>100	-	-
PT5	>100	-	-	>100	-	100
PT6	>100	-	-	66.8	64.0-69.6	100
PT7	>100	-	-	>100	-	100, 50, 25
PT8	>100	-	-	63.4	NR[c]	100, 25
PT9	>100	-	-	>100	-	100
PT10	>100	-	-	65.5	62.3-68.8	100
PT11	>100	-	-	100	NR[c]	100, 50, 25
PT12	>100	-	-	>100	-	100, 50
PT13	>100	-	-	>100	-	100
PT14	>100	-	-	>100	-	100
PT15	>100	-	100	81.4	75.7-87.5	100
PT16	>100	-	-	66.0	58.0-75.1	100, 50
PT17	>100	-	-	69.4	62.1-77.6	100
PT18	>100	-	-	62.0	58.7-65.4	100
PT19	>100	-	-	62.6	NR[c]	100, 50, 25
PT20	>100	-	-	62.3	55.8-69.5	100
PT21	>100	-	-	59.3	54.8-64.2	100, 50
PT22	>100	-	-	61.6	57.5-65.9	100
PT23	>100	-	100	65.6	61.7-69.6	100
PT24	>100	-	-	66.8	NR[c]	100, 25
PT25	>100	-	-	68.0	NR[c]	100
SQ1	>100	-	-	32.6	NR[c]	100, 50
SQ2	98.6	86.4-112.7	100	34.4	NR[c]	100, 50

[a] Percent of water quality adjusted porewater sample.
[b] Dilution of pore water significantly toxic ($\alpha \leq 0.05$) relative to reference sample.
[c] 95% confidence limits not reliable.

sample, stations 0B5, OB7, OB14, OB20, OB22 and OB24 at Jackson Park site, stations PT15 and PT23 at the Port Hadlock site, and station SQ2 in Sequim Bay were significantly toxic.

EC_{50} values were calculable for 22% (12 of 55) of the samples. The remaining 78% of the samples were not toxic enough for the calculation of an EC_{50}, which would be >100%. Only one of the samples, from station OB12, had an EC_{50}<25%, indicating that it would still be toxic at a dilution <25% (Table 3). The toxicity results for the quality assurance replicate samples were practically identical. Stations OB1/OB27 and PT13/PT26 were non-toxic, with EC_{50}>100%. Replicates OB8/OB26 were significantly toxic at a 50% dilution and EC_{50} values were 54.4 and 57.3, respectively, with overlapping 95% confidence limits.

Sea Urchin Embryological Development Toxicity Test

As for the fertilization test, samples used in the sea urchin embryological development tests were considered toxic when their effect was significantly higher ($\alpha \leq 0.05$) than that of the reference sample and the percent normal embryos was below the detectable significance criteria (DSC) at $\alpha \leq 0.05$. The urchin embryological development test was more sensitive than the fertilization test. Only three stations, all at the Port Hadlock site (PT1, PT2 and PT4), did not exhibit toxicity in the undiluted pore water (100% strength) (Figures 11 and 12, Table 3, Appendix D2). At the Port Hadlock site, the samples from stations PT7, PT8, PT11, PT19 and PT24 were the most toxic, causing an adverse effect at the 25% dilution. Samples from stations PT12, PT14 and PT21 were toxic at the 50% dilution and the remaining 14 samples were toxic when undiluted (100%). Higher levels of toxicity occurred in the samples from Jackson Park site, with stations OB6, OB15 and OB22 toxic at a 25% dilution, stations OB4, OB5, OB8, OB10, OB11, OB12, OB13, OB14, OB18, OB19 and OB23 toxic at a 50% dilution, and the remaining 11 stations toxic in the undiluted porewater sample. EC_{50} values were calculable for 82% of the samples (45 of 55), and the EC_{50} for the ten remaining samples would be >100% (Table 3).

The toxicity results for the quality assurance replicate samples were more variable with this method than with the fertilization test, but still close to each other. Samples OB1/OB27 were toxic at 100% sample and had EC_{50} values of 71.3 and 65.2, respectively. Samples OB8/OB26 were significantly toxic at 50% dilution and EC_{50} values of 35.2 and 35.7, respectively. Samples PT13/PT26 were toxic at 100%, but EC_{50} values >100 and 68.0% were significantly different. The difference only occurred due to higher mortality in the 100% sample of PT26, whereas mortality rates in 50 and 25% dilutions were very similar (Appendix D2). The significant difference between samples is usually considered more important than the EC_{50} values for porewater testing, since they provide more reliable data than an EC_{50} calculated based on such small dilution series.

Reference Toxicant Test Results

The EC_{50} values for the reference toxicant, SDS, were 4.8 (4.4-5.1) in the fertilization test, and 3.8 (3.6-4.0) in the embryological development test. These values are within the acceptable limits for SDS toxicity tests, according to our laboratory's control chart. This indicates that the organisms used for these tests were within the usual and acceptable range of sensitivity.

Porewater Quality Measurements

The values obtained for the various water quality parameters measured prior to porewater toxicity tests are presented in Appendix D3. Salinity ranged from 14 to 32 $^\circ/_{\circ\circ}$. Only 15 samples required salinity adjustment to satisfy the test salinity requirement of 30 ± 1 $^\circ/_{\circ\circ}$. The original salinity of ten samples was 32 $^\circ/_{\circ\circ}$ and was adjusted to 30 ± 1 $^\circ/_{\circ\circ}$ by addition of purified deionized water. The salinity of five samples was between 14 and 28 $^\circ/_{\circ\circ}$ and was adjusted using a 122 $^\circ/_{\circ\circ}$ brine made from seawater with 10% reference pore water added.

The initial dissolved oxygen (DO) of only one sample (SQ2) was <80% saturation (75.1%), but was still considered sufficiently high to be used in the tests without prior aeration, which might remove some of the toxicants of concern. The pH of all samples ranged from 7.37 to 7.78. Total ammonia (NH_4) ranged from 0.006 (PT1) to 3.5 (SQ1) mg/L, and unionized ammonia (NH_3) ranged from 0.00 to 45.9 (OB15) µg/L. These values are below the toxic range for fertilization and embryological development tests with *A. punctulata* (Carr *et al.*, 1996b)

Sulfide concentrations were below detection limit (< 0.005 mg/L) in all of the samples except SQ1 and SQ2 both of which had sulfide concentrations of 0.011 mg/L. The higher sulfide level and low DO in the samples from Sequim Bay suggest a high organic load, which could be associated with higher levels of a variety of contaminants, therefore contributing to the toxic effects of those samples to the sea urchin embryos. No chemical measurements were made with these samples to corroborate this hypothesis.

Sediment Chemistry and Grain Size Distribution

Extensive chemical and particle size distribution analyses were conducted with selected sediments from the most toxic stations (Appendices D4 and D5). Particle size distribution varied among samples, from fine sediments with only 10 to 12% sand (station OB16, OB23) to coarse sediments with >80% sand (stations OB6, PT7, PT12, PT16, PT19). The only ordnance compound in measurable amounts was picric acid, found in concentrations ≤1.0 mg/kg in total sediment (dry weight). Total butyltins occurred in some samples, reaching levels up to 8 µg/kg sediment dry weight.

Concentrations of total PCBs, and selected organochlorinated compounds, PAHs and metals were compared to the available Sediment Quality Guidelines (SQGs) (Long *et al.*, 1995; Mac Donald *et al.*, 1996). Stations exceeding the Effects Range-Low (ERL) and Threshold Effects Level (TEL) are presented in Table 4. The stations with most SQG exceedances were OB18 and OB8, with 20 and 18 exceedances, respectively. Stations OB22 and OB23 had 17 exceedances each. Station OB22 was the only one with a chemical (phenanthrene) exceeding the Probable Effects Level (PEL). Stations OB15 and OB16 had nine SQG exceedances each, and OB12 had four. The stations from Port Hadlock site were less contaminated, with only four SQG exceedances at station PT11, two at PT16 and one at PT19. The high number of SQG exceedances at stations OB8, OB18 and OB22, including an exceedance of the PEL for phenanthrene at station OB22, agrees with the high toxicity observed for those samples (Figure 11).

Table 4. Threshold-effects level (TEL), probable effects level (PEL), and the effects range low and median (ERL and ERM, respectively) values for key contaminants and stations exceeding those values.

Contaminant	TEL	PEL	ER-L	ER-M	Stations exceeding TEL or ER-L
Pesticides and Polychlorinated Biphenyls (µg/kg)					
Chlordane	2.26	4.79	0.5	6	OB8, OB12, PT11, PT16
Dieldrin	0.72	4.3	0.02	8	OB8, OB15, OB16, OB18, OB23, PT11, PT19
p,p' - DDD	1.22	7.81	2	20	
p,p' - DDE	2.07	374	2.2	27	
p,p' - DDT	1.19	4.77	1	7	OB15, OB18, OB23, PT11
Total DDT	3.89	51.7	1.58	46.1	OB15, OB18, OB23, PT11
Total PCBs	21.6	189	22.7	180	OB8, OB15, OB16, OB18, OB22, OB23
Polycyclic Aromatic Hydrocarbons (µg/kg)					
Acenaphthene	6.71	88.9	16	500	OB22
Acenaphthylene	5.87	128	44	640	OB8, OB15, OB16, OB18, OB22, OB23
Anthracene	46.9	245	85.3	1100	OB18, OB22
Fluorene	21.2	144	19	540	OB18
Naphthalene	34.6	391	160	2100	
2-Methyl Naphthalene	20.2	201	70	670	
Phenanthrene	86.7	544	240	1500	OB22[a]
∑ LMW PAHs[b]	312	1442	552	3160	OB22
Benz(a)-anthracene	74.8	693	261	1600	OB8, OB18, OB22, OB23
Benzo(a)pyrene	88.8	763	430	1600	OB8, OB12, OB18, OB22, OB23
Chrysene	108	846	384	2800	OB8, OB12, OB18, OB22, OB23
Dibenzo(a,h)-anthracene	6.22	135	63.4	260	OB8, OB12, OB15, OB16, OB18, OB22, OB23
Fluoranthene	113	1494	600	5100	OB8, OB18, OB22, OB23, PT16
Pyrene	153	1398	665	2600	OB8, OB18, OB22, OB23
∑ HMW PAHs[c]	655	6676	1700	9600	OB8, OB18, OB22, OB23
Total PAHs[d]	1684	16,770	4022	44,792	OB18,OB22

Table 4. Continued

Trace Elements (mg/kg)					
As	7.24	41.6	8.2	70	OB8, OB16, OB18, OB23
Cd	0.68	4.21	1.2	9.6	OB8, OB16, OB18, OB22, OB23
Cr	52.3	160	81	370	OB8
Cu	18.7	108	34	270	OB8, OB15, OB16, OB18, OB22, OB23
Pb	30.2	112	46.7	218	OB8, OB15, OB16, OB18, OB22, OB23
Hg	0.13	0.7	0.15	0.71	OB8, OB15, OB16, OB18, OB22, OB23
Zn	124	271	150	410	OB8

[a] Above PEL.
[b] Sum of the following low molecular weight PAHs; acenaphthene, acenaphthylene, anthracene, fluorene, 2-methylnaphthalene, naphthalene and phenanthrene
[c] Sum of the following high molecular weight PAHs; benz(a)anthracene, benzo(a)pyrene, chrysene, dibenzo(a,h,)anthracene, fluoranthene and pyrene
[d] Sum of high and low molecular weight PAHs described above.

Toxicity Identification Evaluation (TIE) Procedures - Phase I

Fresh and frozen pore water comparison

Based on the toxicity test results with pore water and the chemistry data, three adjacent sites from Ostrich Bay (OB8, OB18 and OB22) which were toxic to both the fertilization and embryological development tests, were selected to be combined and submitted to a TIE procedure. Initially, a comparison of the toxicity and chemistry of fresh and previously frozen pore water was conducted. The EC_{50} values for fresh and frozen pore water in the sea urchin fertilization test were 29.2% (15.7-54.3) and 44.7% (27.9-71.6), respectively. The overlapping 95% confidence intervals from both tests, presented in parenthesis, indicates that there was no significant difference between the two results. For the sea urchin embryological development test, the EC50 values were identical 35.4 (95% confidence limit not reliable) for the fresh and frozen porewater samples. The complete data set for these tests is in Appendix E1, and water quality measurements are on Appendix E2.

A summary of chemical measurements made in the fresh and frozen porewater samples, including the sum of ordnance compounds, butyltins and PCBs, as well as PAHs and metals that were in measurable amounts, is presented in Table 5. The complete list of chemical measurements made with these porewater samples is in Appendix E3. Concentrations of organic chemicals varied only slightly between the frozen and fresh sample, with higher levels of some PAHs in the frozen pore water, suggesting some loss in the fresh sample between the extraction

Table 5: Summary of chemical measurements in fresh and frozen pore water.

Chemical	Concentration in frozen and fresh pore water (µg/L)	
	Frozen	**Fresh**
Ordnance Compounds	ND[a]	ND[a]
Butyltins	0.118	0.120
Polychlorinated Biphenyls (PCBs)	ND[a]	ND[a]
Polyaromatic Hydrocarbons (PAHs) above detection limit		
Phenol	1.5	1.5
Naphthalene	0.03	0.03
Diethyl Phthalate	0.2	0.2
Phenanthrene	0.05	0.05
Di-n-butyl Phthalate	4.3	4.4
Butyl Benzyl Phthalate	0.06	0.05
Bis(2-ethylhexyl) Phthalate	3	0.2
Indeno(1,2,3-cd)pyrene	0.07	0
Dibenz(a,h)anthracene	0.08	0
Benzo(g,h,i)perylene	0.07	0
Metals		
Arsenic	4.3	8.0
Cadmium	0.04	0.08
Chromium	ND	0.6
Copper	0.2	0.3
Lead	0.06	0.14
Zinc	0.6	1.1

[a] Not detectable.

and analysis times. The concentrations of metals, on the other hand, dropped to approximately half of the initial concentration after freezing. The toxicity test results, however, showed that there were no significant change in toxicity after freezing and thawing the sample. Therefore, frozen samples were used for the application of the TIE treatments.

The reference toxicant (SDS) test conducted concurrently with the fresh and frozen sample comparison, resulted in EC_{50} values of 4.5 mg/L (4.1-5.0) and 7.1 mg/L (95% confidence limit not reliable) for the fertilization and embryological development tests, respectively. This was within the acceptable sensitivity range for the fertilization test, but indicated slightly lower sensitivity than usual for the embryological development test, for which the upper acceptable limit for the EC_{50} would be 6.8 mg/L. However, it was considered acceptable for the purpose of this particular test, which was of comparing two samples treated in different ways (fresh vs. freezing).

<u>TIE procedure</u>

The TIE procedure was applied to full strength pore water as well as to samples diluted to 50% and 25%. Results from the urchin fertilization test showed that the C_{18} column treatment significantly reduced toxicity from the three dilutions, providing strong indication that organic compounds would be responsible for some of the observed toxic effects (Table 6). At the 100% dilution, the addition of EDTA also caused significant reduction of toxicity, suggesting some contribution of metals as causative agents of toxicity. The increase of pH to 9.0 significantly increased toxicity at the three dilutions, but no significant reduction of toxicity was observed with pH reduction. Therefore, ammonia was not considered as a toxicant of concern in those samples for the fertilization endpoint. The complete data set for this experiment is presented in Appendix E4.

The toxicity of the 100% and 50% dilution samples for the TIE procedures employing the embryological development test was still very high after all TIE treatments were applied (see Appendix E5). The 25% dilution samples indicated reduction of toxicity with several treatments: EDTA, C_{18} column, sodium thiosulfate, pH reduction (Table 7). This suggests that several factors were contributing to the toxicity of that sample, including metals, organic compounds and ammonia. The results of the TIE study and of the chemical analysis of the sample used for the TIE indicate that ordnance compounds would not have been responsible for the toxicity observed in that sample.

The reference toxicant tests conducted with SDS concurrently to the TIE procedures presented EC_{50} values of 4.5 mg/L (4.2-4.8) and 3.3 mg/L (3.2-3.4) for the fertilization and embryological development tests, respectively. These values indicate that the test organisms were within the acceptable range of sensitivity based on the control charts.

Additional Phase II TIE studies are currently being conducted to better determine the compounds or classes of compounds that are contributing to the observed toxicity in porewater from this composite sample. These studies will involve fractionating the porewater and identifying the most toxic fractions which will be further analyzed chemically to identify the toxicants responsible for the observed toxicity. The results from these studies will be included in a latter report.

Table 6. Toxicity data for sea urchin, *A. punctulata,* fertilization test following TIE procedures.

Treatment	Sample	% Dilution	Mean % Fertilized	Diff.[d]
Baseline	OB[a]	100	22.4	
Baseline	OB	50	40.4	
Baseline	OB	25	74.8	
Baseline	OB	12.5	94	
Baseline	OB	6.25	95.2	
Baseline	REF[b]	100	87.8	
Baseline	REF	50	91.6	
Baseline	REF	25	93	
Baseline	REF	12.5	95.8	
Baseline	REF	6.25	93.8	
Baseline	MFS[c]	100	89.7	
Aeration	OB	100	16.8	
Aeration	OB	50	27.6	
Aeration	OB	25	73.2	
Aeration	MFS	100	83.8	
Filtration	OB	100	30	
Filtration	OB	50	42.6	
Filtration	OB	25	81	
Filtration	MFS	100	83.4	
C18	OB	100	56	**
C18	OB	50	73.8	**
C18	OB	25	89.2	**
C18	MFS	100	84.2	
EDTA	OB	100	44.6	*
EDTA	OB	50	42.6	
EDTA	OB	25	81	
EDTA	MFS	100	93.8	
Na thiosulfate	OB	100	32.8	
Na thiosulfate	OB	50	32	
Na thiosulfate	OB	25	73.8	
Na thiosulfate	MFS	100	91.6	
pH 7.2	OB	100	25	
pH 7.2	OB	50	26	
pH 7.2	OB	25	39.2	**
pH 7.2	REF	100	60.2	
pH 7.2	REF	50	84	
pH 7.2	REF	25	82.4	
pH 7.2	MFS	100	85.8	
pH 8.0	OB	100	23	
pH 8.0	OB	50	29.2	

Table 6. Continued

Treatment	Sample	% Dilution	Mean % Fertilized	Diff.[d]
pH 8.0	OB	25	67.2	
pH 8.0	REF	100	38	
pH 8.0	REF	50	84.6	
pH 8.0	REF	25	90.4	
pH 8.0	MFS	100	92.8	
pH 9.0	OB	100	0	**
pH 9.0	OB	50	0	**
pH 9.0	OB	25	13.2	**
pH 9.0	REF	100	11.8	
pH 9.0	REF	50	59.4	
pH 9.0	REF	25	75.6	
pH 9.0	MFS	100	89.4	

Table 7. Data for sea urchin embryological development test following TIE procedures.

Treatment	Sample	% Dilution	Mean % Normal	Sig. Diff.[d]
Baseline	OB[a]	25	7.00	
Baseline	REF[b]	25	89.40	
Baseline	MFS[c]	100	89.40	
Filtration	OB	25	13.00	
Filtration	MFS	100	91.80	
Aeration	OB	25	3.80	
Aeration	MFS	100	87.20	
EDTA	OB	25	80.60	**
EDTA	MFS	100	85.80	
C18	OB	25	54.40	**
C18	MFS	100	90.80	
Na thiosulfate	OB	25	46.00	**
Na thiosulfate	MFS	100	86.20	
pH 7.2	OB	25	90.00	**
pH 7.2	REF	25	91.80	
pH 7.2	MFS	100	87.20	
pH 8.0	OB	25	34.80	**
pH 8.0	REF	25	89.00	
pH 8.0	MFS	100	89.60	
pH 9.0	OB	25	0.00	
pH 9.0	REF	25	88.20	
pH 9.0	MFS	100	91.60	

[a] Pore water from site selected for TIE, from Ostrich Bay
[b] Reference pore water, from Redfish Bay, Texas
[c] Millipore filtered seawater
[d] Significantly different from Ostrich Bay baseline toxicity, * indicates significant difference at alpha ≤ 0.05 and ** indicates significant difference at alpha ≤ 0.01.

CONCLUSIONS

- There was a large range in toxicity among the different ordnance compounds tested and a large range in response for the different tests for a particular compound.

- The most toxic ordnance compounds, tetryl and 1,3,5-TNB, were also the most degradable. Therefore, these chemicals are expected to be short-lived in nature, and environmental impacts would not be expected in areas that are not currently subject to chronic inputs of these chemicals.

- Although toxicity, as measured by the highly sensitive sea urchin embryological development test, was observed at the majority of stations at both Jackson Park and the Port Hadlock sites, it is highly unlikely to be caused by ordnance compounds. The only ordnance compound detected in sediments from the most toxic stations was picric acid, in low concentrations of ≤ 1 mg/kg sediment dry weight, and it was not detected at one of the most toxic stations to sea urchin embryos (PT7).

- No ordnance compounds were detected in the porewater sample used for the TIE study which indicates that explosives were not responsible for the toxicity observed in this composite sample from the most toxic stations.

- The Phase I TIE procedures indicated that organic chemicals (PAHs, PCBs, pesticides), and metals to a smaller extent, were the main causative agents of the toxicity observed in the sea urchin fertilization test.

- The Phase I TIE procedures indicated that several classes of chemicals, including organic chemicals (PAHs, PCBs, pesticides), metals and ammonia, were the main causative agents of the toxic effect in the sea urchin embryological development test.

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Appendix A

Complete data set for toxicity tests with ordnance compounds

Appendix A1. Sea urchin, *Arbacia punctulata*, toxicity data for fertilization test with ordnance compounds.

Appendix A2. Sea urchin, *Arbacia punctulata*, toxicity data for embryological development test with ordnance compounds.

Appendix A3. Macro-alga, *Ulva fasciata*, toxicity test data for ordnance compounds: germination endpoint.

Appendix A4. Macro-alga, *Ulva fasciata*, toxicity test data for ordnance compounds: germling length endpoint.

Appendix A5. Macro-alga, *Ulva fasciata*, toxicity test data for ordnance compounds: germling cell number endpoint.

Appendix A6. Polychaete, *Dinophilus gyrociliatus*, toxicity test data for ordnance compounds: survival.

Appendix A7. Polychaete, *Dinophilus gyrociliatus*, toxicity test data for ordnance compounds: reproduction.

Appendix A8. Redfish, *Sciaenops ocellatus*, toxicity test survival data for ordnance compounds.

Appendix A9. Mysid, *Mysidopsis bahia*, toxicity test survival data for ordnance compounds.

Appendix A10. Reference toxicant (SDS) data for all tests conducted concurrently with ordnance compound toxicity tests.

Appendix A1. Sea urchin, *Arbacia punctulata,* toxicity data for fertilization test with ordnance compounds.

Date	Sample	Initial concentration (mg/L)	% Fertilized: Replicate number 1	2	3	4	5	Mean % fertilized	SD	Significantly different from control[d]	EC_{50} (mg/L)
9/4/98	MFS[a]	100	81	98	91	92	80	89.7	5.81		
		100	91	87	95	88	94				
9/4/98	MFS-MEOH[b]	100	97	99	94	94	94	95.6	2.30		
9/4/98	MFS-Teflon[c]	100	91	94	98	92	98	94.6	3.29		
9/4/98	2,4-DNT	75.2	35	45	51	31	34	39.2	8.44	**	68.0 (61.2-75.6)
	2,4-DNT	38.6	92	86	85	92	88	88.6	3.29		
	2,4-DNT	18.4	93	95	90	93	97	93.6	2.61		
9/4/98	2,6-DNT	84.5	73	52	67	46	55	58.6	11.10	**	>84.5
	2,6-DNT	45.2	49	57	71	70	70	63.4	9.91	**	
	2,6-DNT	23.3	92	87	72	88	93	86.4	8.44		
9/4/98	2,4,6-TNT	103.1	81	72	81	76	68	75.6	5.68	**	>103.1
	2,4,6-TNT	27.7	93	94	93	94	90	92.8	1.64		
9/4/98	RDX	26.4	93	95	81	88	87	88.8	5.50		>74.7
	RDX	74.7	86	90	82	91	90	87.8	3.77		
9/4/98	1,3-DNB	315.2	52	45	78[e]	27	28	38	12.46	**	258.0 (216.6-308.9)
	1,3-DNB	109.8	88	81	86	77	81	82.6	4.39	**	
	1,3-DNB	84.3	96	90	94	82	85	89.4	5.90		
9/4/98	1,3,5-TNB	264.1	2	0	1	0	0	0.6	0.89	**	84.3 (76.4-93.0)
	1,3,5-TNB	141.8	25	24	29	16	22	23.2	4.76	**	
	1,3,5-TNB	47.7	64	71	85	60	61	68.2	10.33	**	
	1,3,5-TNB	35.2	94	96	97	88	91	93.2	3.70		
9/4/98	Tetryl	9.9	4	2	0	1	1	1.6	1.52	**	2.96 (2.51-3.49)
	Tetryl	6.9	17	33	20	21	14	21	7.25	**	
	Tetryl	4.4	67	41	24	64	26	44.4	20.38	**	
	Tetryl	2.2	74	60	67	56	65	64.4	6.88	**	
	Tetryl	1.1	64	58	71	78	40	62.2	14.50	**	
	Tetryl	0.6	78	39[e]	72	74	91	78.75	8.54	**	

Appendix A1. Continued.

Date	Sample	Initial concentration (mg/L)	% Fertilized							Significantly different from control[d]	EC_{50} (mg/L)
			Replicate number					Mean			
			1	2	3	4	5	% fertilized	SD		
9/4/98	Picric Acid	**704.4**	1	1	1	1	0	**0.8**	0.45	**	
	Picric Acid	**352.3**	41	53	58	60	69	**56.2**	10.28	**	**349.3 (321.0-380.2)**
	Picric Acid	**177.6**	76	88	84	88	88	**84.8**	5.22		
	Picric Acid	**92.2**	83	92	94	92	86	**89.4**	4.67		
	Picric Acid	**49.6**	92	89	95	93	95	**92.8**	2.49		

[a] MFS: millipore filtered seawater; [b] MFS with methanol carrier at highest concentration used in test solutions; [c] MFS filtered through Teflon filter, to analyse potential eeffects of filter; [d] ** indicates significant difference at alpha $\leq$0.01; [e] data detected as outlier and not included in statistical analyses.

Appendix A2. Sea urchin, *Arbacia punctulata*, toxicity data for embryological development test with ordnance compounds.

Date	Sample	Initial concentration (mg/L)	% Normal Development: Replicate number 1	2	3	4	5	Mean % normal	SD	Significantly different from control[d]	EC_{50} (mg/L)
9/4/98	MFS[a]	**100**	90	86	90	88	91	**89**	2.00		
9/4/98	MFS-MEOH[b]	**100**	92	92	90	86	84	**88.8**	3.63		
9/4/98	MFS-Teflon[c]	**100**	87	87	90	92	83	**87.8**	3.42		
10/7/98	MFS[a]	**100**	85	89	87	85	80	**85.2**	3.35		
10/19/98	MFS[a]	**100**	83	80	76	82	79	**80**	2.74		
9/4/98	2,4-DNT	**75.2**	0	0	0	0	0	**0**	0.00	**	
	2,4-DNT	**38.6**	70	88	86	23[e]	83	**81.75**	8.10	*	**51.38 (49.33-53.51)**
	2,4-DNT	**18.4**	87	89	88	90		**88.5**	1.29		
	2,4-DNT	**8.9**	88	88	88	86		**87.5**	1.00		
9/4/98	2,6-DNT	**21.8**	0	0	0	0	0	**0**	0.00	**	**6.68**
	2,6-DNT	**10.3**	0[e]	11	31	19	13	**18.5**	9.00	**	**(6.09-7.32)**
	2,6-DNT	**5**	77	60	59	49	63	**61.6**	10.09	**	
9/4/98	2,4,6-TNT	**39.2**	0	0	0	0	0	**0**	0.00	**	
	2,4,6-TNT	**19.1**	0	2	0	0	1	**0.6**	0.89	**	**11.63 (10.81-12.52)**
	2,4,6-TNT	**9.1**	83	81	70	77	79	**78**	5.00	**	
	2,4,6-TNT	**2.1**	91	93	87	87	90	**89.6**	2.61		
9/4/98	RDX	**74.7**	91	88	81	87	95	**88.4**	5.18		**>74.7**
	RDX	**26.4**	88	93	91	89	90	**90.2**	1.92		
9/4/98	1,3-DNB	**315.2**	0	0	0	0	0	**0**	0.00	**	
	1,3-DNB	**109.8**	0	0	0	0	0	**0**	0.00	**	**92.06**
	1,3-DNB	**84.3**	75	63	63	74	62	**67.4**	6.50	**	**(nr)**
10/19/98	1,3,5-TNB	**2.019**	0	0	0	0	0	**0**	0.00	**	
	1,3,5-TNB	**1.096**	72	71	72	69	72	**71.2**	1.30	**	**1.28**
	1,3,5-TNB	**0.476**	72	74	79	77	75	**75.4**	2.70	*	**(1.20-1.36)**
	1,3,5-TNB	**0.237**	82	81	80	75	75	**78.6**	3.36		
	1,3,5-TNB	**0.122**	86	85	76	82	82	**82.2**	3.90		

Appendix A2. Continued.

Date	Sample	Initial concentration (mg/L)	% Normal Development							Significantly different from control[d]	EC_{50} (mg/L)
			Replicate number					Mean			
			1	2	3	4	5	% normal	SD		
10/7/98	Tetryl	**0.162**	0	0	0	0	0	**0**	0.00	**	
	Tetryl	**0.083**	53	41	38	37	29	**39.6**	8.71	**	**0.08 (0.07-0.08)**
	Tetryl	**0.036**	86	85	81	83	85	**84**	2.00		
	Tetryl	**0.014**	93	85	87	88	89	**88.4**	2.97		
9/4/98	Picric Acid	**704.4**	0	0	0	0	0	**0**	0.00	**	
	Picric Acid	**352.3**	33[e]	18	13	12	16	**14.75**	2.75	**	**281.22**
	Picric Acid	**177.6**	90	84	88	95	89	**89.2**	3.96		**(267.03-296.15)**

[a]MFS: millipore filtered seawater; [b] MFS with methanol carrier at highest concentration used in test solutions; [c] MFS filtered through Teflon filter, to analyse potential eeffects of filter; [d] * indicates significant difference at alpha ≤0.05, and ** indicates significant difference at alpha ≤0.01;
[e] data detected as outlier and not included in statistical analyses.

Appendix A3. Macro-alga, *Ulva fasciata*, toxicity test data for ordnance compounds: germination endpoint.

Date	Sample	Initial concentration (mg/L)	% Germinated: Replicate number 1	2	3	4	5	Mean	SD	Contro	% Difference from control	Significantly different from control[d]	EC_{50} (mg/L)
1/15/99	Control[a]	**100**	84	79	87	82	88	**84**	3.67	100	0		
1/15/99	PW[b]	**100**	82	85	80	80	87	**82.8**	3.11	99	1		
1/15/99	2,4-DNT	**3.474**	7	23	9	12	16	**13.4**	6.35	16	84	**	**2.48 (2.36-2.60)**
	2,4-DNT	**1.773**	66	67	76	69	78	**71.2**	5.45	85	15	**	
	2,4-DNT	**0.936**	90	93	95	88	96	**92.4**	3.36	110	-10		
	2,4-DNT	**0.475**	89	95	94	90	91	**91.8**	2.59	109	-9		
1/15/99	2,6-DNT	**19.738**	0	0	0	0	0	**0**	0.00	0	100	**	**6.70 (6.15-7.31)**
	2,6-DNT	**9.505**	20	22	20	23	16	**20.2**	2.68	24	76	**	
	2,6-DNT	**4.662**	66	59	70	67	62	**64.8**	4.32	77	23	**	
	2,6-DNT	**2.247**	99	85	90	92	92	**91.6**	5.03	109	-9		
	2,6-DNT	**1.202**	94	97	98	99	97	**97**	1.87	115	-15		
1/15/99	2,4,6-TNT	**3.438**	7	4	14	15	7	**9.4**	4.83	11	89	**	**2.54 (2.47-2.61)**
	2,4,6-TNT	**1.741**	84	82	80	88	80	**82.8**	3.35	99	1		
	2,4,6-TNT	**0.898**	77	81	94	95	91	**87.6**	8.11	104	-4		
	2,4,6-TNT	**0.43**	92	97	93	95	95	**94.4**	1.95	112	-12		
	2,4,6-TNT	**0.208**	98	98	93	100	95	**96.8**	2.77	115	-15		
1/15/99	RDX	**31.301**	0	0	0	0	0	**0**	0.00	0	100	**	**12.0 (11.5-12.4)**
	RDX	**15.689**	1	6	3	5	7	**4.4**	2.41	5	95	**	
	RDX	**9.189**	86	70	82	78	82	**79.6**	6.07	95	5		
	RDX	**4.986**	83	78	82	83	86	**82.4**	2.88	98	2		
1/15/99	1,3-DNB	**2.515**	0	0	0	0	0	**0**	0.00	0	100	**	**0.85 (0.80-0.90)**
	1,3-DNB	**1.255**	9	5	3	4	5	**5.2**	2.28	6	94	**	
	1,3-DNB	**0.651**	60	79	73	77	65	**70.80**	8.07	84	16	**	
	1,3-DNB	**0.313**	95	94	94	90	92	**93**	2.00	111	-11		
	1,3-DNB	**0.208**	92	91	92	90	94	**91.8**	1.48	109	-9		
1/15/99	1,3,5-TNB	**0.186**	14	0	0	3	2	**3.8**	5.85	5	95	**	**0.08 (0.07-0.09)**
	1,3,5-TNB	**0.093**	15	26	35	27	18	**24.2**	7.92	29	71	**	
	1,3,5-TNB	**0.046**	78	85	81	90	86	**84**	4.64	100	0		
	1,3,5-TNB	**0.029**	94	95	95	96	89	**93.8**	2.77	112	-12		

Appendix A3. Continued.

Date	Sample	Initial concentration (mg/L)	% Germinated: Replicate number 1	2	3	4	5	Mean	SD	Contro	% Difference from control	Significantly different from control[d]	EC_{50} (mg/L)
1/15/99	Tetryl	**1.003**	0	0	0	0	0	**0**	0.00	0	100	**	**0.67 (0.65-0.70)**
	Tetryl	**0.503**	81	82	77	74	71	**77**	4.64	92	8		
	Tetryl	**0.25**	90	81	86	88	82	**85.4**	3.85	102	-2		
	Tetryl	**0.098**	93	99	96	97	98	**96.6**	2.30	115	-15		
1/15/99	Picric Acid	**662.742**	0	4	3	5	2	**2.8**	1.92	3	97	**	**415 (388.8-442.9)**
	Picric Acid	**336.099**	9[c]	57	68	65	69	**64.75**	5.44	77	23	**	
	Picric Acid	**169.168**	87	81	88	90	86	**86.4**	3.36	103	-3		
	Picric Acid	**92.09**	94	90	89	94	95	**92.4**	2.70	110	-10		

[a] Control = Millipore filtered seawater with 10% pore water added as nutrient supply for the zoospores; [b] PW = pore water only, as an additional control;
[c] data detected as outlier and not included in statistical analyses; [d] ** indicates significant difference at alpha $\leq$0.01

Appendix A4. Macro-alga, *Ulva fasciata,* toxicity test data for ordnance compounds: germling length endpoint.

Date	Sample	Initial concentration (mg/L)	Germling length (um) Replicate number 1	2	3	4	5	Mean	SD	%Control	% Difference from control	Significantly different from control[c]	EC_{50} (mg/L)
1/15/99	Control[a]	**100**	45.63	39.04	38.53	38.53	41.83	**40.71**	3.07	100			
1/15/99	PW[b]	**100**	24.84	24.84	24.59	25.35	24.34	**24.79**	0.38	61			
1/15/99	2,4-DNT	**3.474**	9.63	7.35	7.35	8.62	9.89	**8.57**	1.21	21	79	**	
	2,4-DNT	**1.773**	15.72	21.04	21.55	24.59	22.31	**21.04**	3.27	52	48	**	**1.71**
	2,4-DNT	**0.936**	34.73	26.87	28.90	28.14	27.89	**29.30**	3.12	72	28	**	**(1.46-2.00)**
	2,4-DNT	**0.475**	32.45	37.01	31.94	32.19	32.96	**33.31**	2.10	82	18	**	
1/15/99	2,6-DNT	**19.738**	0	0	0	0	0	**0.00**	0.00	0	100	**	
	2,6-DNT	**9.505**	7.61	6.34	7.86	7.86	6.84	**7.30**	0.68	18	82	**	**2.87**
	2,6-DNT	**4.662**	14.45	13.94	12.68	16.22	15.46	**14.55**	1.37	36	64	**	**(2.32-3.55)**
	2,6-DNT	**2.247**	26.36	18.25	20.53	26.36	26.11	**23.52**	3.86	58	42	**	
	2,6-DNT	**1.202**	24.34	26.87	38.03	30.42	27.38	**29.41**	5.28	72	28	**	
1/15/99	2,4,6-TNT	**3.438**	7.86	6.34	8.37	8.11	8.62	**7.86**	0.90	19	81	**	
	2,4,6-TNT	**1.741**	15.46	13.44	14.70	16.22	15.72	**15.11**	1.08	37	63	**	**0.76**
	2,4,6-TNT	**0.898**	23.83	17.24	23.32	19.77	16.98	**20.23**	3.25	50	50	**	**(0.44-1.30)**
	2,4,6-TNT	**0.43**	19.77	26.11	23.32	23.58	22.82	**23.12**	2.26	57	43	**	
	2,4,6-TNT	**0.208**	24.08	21.80	25.35	21.80	29.15	**24.44**	3.05	60	40	**	
1/15/99	RDX	**31.301**	0	0	0	0	0	**0.00**	0.00	0	100	**	**8.14**
	RDX	**15.689**	6.34	5.83	7.86	6.84	6.34	**6.64**	0.77	16	84	**	**(7.07-8.14)**
	RDX	**9.189**	19.27	14.96	18.76	21.29	19.01	**18.66**	2.30	46	54	**	
	RDX	**4.986**	28.90	28.39	28.39	28.39	29.66	**28.75**	0.56	71	29	**	
1/15/99	1,3DNB	**2.515**	0	0	0	0	0	**0.00**	0.00	0	100	**	
	1,3DNB	**1.255**	7.86	6.84	7.61	6.59	7.35	**7.25**	0.53	18	82	**	**0.41**
	1,3DNB	**0.651**	12.93	12.42	10.14	9.89	7.61	**10.60**	2.15	26	74	**	**(0.36-0.47)**
	1,3DNB	**0.313**	22.82	22.31	25.86	24.34	26.62	**24.39**	1.87	60	40	**	
	1,3DNB	**0.208**	29.66	40.81	34.98	37.01	27.38	**33.97**	5.46	83	17	**	
1/15/99	1,3,5-TNB	**0.186**	8.62	0.00	0.00	6.59	6.08	**4.26**	4.00	10	90	**	
	1,3,5-TNB	**0.093**	7.86	7.10	7.35	8.11	7.10	**7.50**	0.46	18	82	**	**0.05**
	1,3,5-TNB	**0.046**	26.62	19.27	18.25	18.25	17.49	**19.98**	3.77	49	51	**	**(0.05-0.06)**
	1,3,5-TNB	**0.029**	40.31	38.03	37.01	38.03	42.84	**39.24**	2.35	96	4		

Appendix A4. Continued

Date	Sample	Initial concentration (mg/L)	Germling length (um)									Significantly different from control[c]	EC_{50} (mg/L)
			Replicate number					Mean	SD	%Control	% Difference from control		
			1	2	3	4	5						
1/15/99	Tetryl	**1.003**	0	0	0	0	0	**0.00**	0.00	0	100	**	**0.34 (0.31-0.39)**
	Tetryl	**0.503**	17.49	15.21	12.42	12.17	15.97	**14.65**	2.31	36	64	**	
	Tetryl	**0.25**	34.48	27.38	23.32	25.86	25.86	**27.38**	4.23	67	33	**	
	Tetryl	**0.098**	31.69	38.03	42.08	42.84	39.80	**38.89**	4.45	96	4		
1/15/99	Picric Acid	**662.742**	0	7.61	6.59	7.35	8.11	**5.93**	3.36	15	85	**	**94.4 (74.1-120.4)**
	Picric Acid	**336.099**	11.41	7.86	9.13	8.11	7.35	**8.77**	1.61	22	78	**	
	Picric Acid	**169.168**	7.35	8.11	15.46	12.68	11.66	**11.05**	3.35	27	73	**	
	Picric Acid	**92.09**	24.08	18.00	16.22	25.35	21.04	**20.94**	3.88	51	49	**	

[a] Control = Millipore filtered seawater with 10% pore water added as nutrient supply for the zoospores; [b] PW = pore water only, as an additional control;

[c] ** indicates significant difference at alpha $\leq$0.01.

Appendix A5. Macro-alga, *Ulva fasciata*, toxicity test data for ordnance compounds: germling cell number endpoint.

Date	Sample	Initial concentration (mg/L)	Germling cell number									Significantly different from control[c]	EC_{50} (mg/L)
			Replicate number					Mean	SD	%Control	% Difference from control		
			1	2	3	4	5						
1/15/99	Control[a]	**100**	4.2	3.9	3.7	4.1	4.3	**4.0**	0.24	100			
1/15/99	PW[b]	**100**	2.8	2.4	2.7	2.8	2.5	**2.6**	0.18	65			
1/15/99	2,4-DNT	**3.474**	1.2	1.0	1.0	1.0	1.2	**1.1**	0.11	27	73	**	
	2,4-DNT	**1.773**	2.1	2.3	2.5	2.6	2.3	**2.4**	0.19	58	42	**	**2.09**
	2,4-DNT	**0.936**	3.9	3.2	3.1	3.2	3.5	**3.4**	0.33	84	16	**	**(1.77-2.48)**
	2,4-DNT	**0.475**	3.2	3.2	3.1	2.9	3.0	**3.1**	0.13	76	24	**	
1/15/99	2,6-DNT	**19.738**	0.0	0.0	0.0	0.0	0.0	**0.0**	0.00	0	100	**	
	2,6-DNT	**9.505**	1.0	1.0	1.0	1.0	1.0	**1.0**	0.00	25	75	**	**4.25**
	2,6-DNT	**4.662**	2.2	1.7	1.5	2.1	2.0	**1.9**	0.29	47	53	**	**(3.50-5.15)**
	2,6-DNT	**2.247**	3.5	2.5	2.8	3.1	2.7	**2.9**	0.39	72	28	**	
	2,6-DNT	**1.202**	2.7	3.1	3.7	3.1	2.9	**3.1**	0.37	77	23	**	
1/15/99	2,4,6-TNT	**3.438**	1.1	1.0	1.0	1.0	1.0	**1.0**	0.04	25	75	**	
	2,4,6-TNT	**1.741**	2.0	1.6	1.8	1.8	2.0	**1.8**	0.17	46	54	**	**1.37**
	2,4,6-TNT	**0.898**	3.2	2.3	2.7	2.5	2.4	**2.6**	0.36	65	35	**	**(1.01-1.86)**
	2,4,6-TNT	**0.43**	2.5	2.8	2.6	2.9	2.8	**2.7**	0.16	67	33	**	
	2,4,6-TNT	**0.208**	3.0	2.6	2.7	2.7	3.1	**2.8**	0.22	70	30	**	
1/15/99	RDX	**31.301**	0.0	0.0	0.0	0.0	0.0	**0.0**	0.00	0	100	**	**9.81**
	RDX	**15.689**	1.0	1.0	1.1	1.0	1.0	**1.0**	0.04	25	75	**	**(8.76-10.98)**
	RDX	**9.189**	2.2	1.9	2.4	2.5	2.1	**2.2**	0.24	55	45	**	
	RDX	**4.986**	3.6	2.9	3.2	3.3	3.5	**3.3**	0.27	82	18	**	
1/15/99	1,3-DNB	**2.515**	0.0	0.0	0.0	0.0	0.0	**0.0**	0.00	0	100	**	
	1,3-DNB	**1.255**	1.0	1.0	1.0	1.0	1.0	**1.0**	0.00	25	75	**	**0.45**
	1,3-DNB	**0.651**	1.5	1.5	1.1	1.2	1.2	**1.3**	0.19	32	68	**	**(0.38-0.54)**
	1,3-DNB	**0.313**	2.7	2.6	2.8	2.7	2.7	**2.7**	0.07	67	33	**	
	1,3-DNB	**0.208**	2.9	3.4	3.0	3.1	2.8	**3.0**	0.23	75	25	**	
1/15/99	1,3,5-TNB	**0.186**	1.0	0.0	0.0	1.0	1.0	**0.6**	0.55	15	85	**	
	1,3,5-TNB	**0.093**	1.0	1.0	1.0	1.1	1.0	**1.0**	0.04	25	75	**	**0.06**
	1,3,5-TNB	**0.046**	2.9	2.3	1.9	2.2	2.2	**2.3**	0.37	57	43	**	**(0.05-0.07)**
	1,3,5-TNB	**0.029**	4.0	3.3	3.6	3.3	4.1	**3.7**	0.38	91	9		

Appendix A5. Continued

Date	Sample	Initial concentration (mg/L)	Germling cell number: Replicate number 1	2	3	4	5	Mean	SD	%Control	% Difference from control	Significantly different from control[c]	EC_{50} (mg/L)
1/15/99	Tetryl	**1.003**	0.0	0.0	0.0	0.0	0.0	**0.0**	0.00	0	100	**	**0.40 (0.35-0.45)**
	Tetryl	**0.503**	1.8	1.8	1.4	1.5	2.0	**1.7**	0.24	42	58	**	
	Tetryl	**0.25**	3.9	2.8	2.8	3.0	3.3	**3.2**	0.46	78	22	**	
	Tetryl	**0.098**	3.0	3.6	3.7	4.2	3.8	**3.7**	0.43	91	9		
1/15/99	Picric Acid	**662.742**	0.0	1.0	1.0	1.0	1.1	**0.8**	0.46	20	80	**	**118 (100.9-138.0)**
	Picric Acid	**336.099**	1.6	1.0	1.0	1.1	1.0	**1.1**	0.26	28	72	**	
	Picric Acid	**169.168**	1.0	1.0	1.9	1.5	1.5	**1.4**	0.38	34	66	**	
	Picric Acid	**92.09**	2.6	2.2	2.2	2.6	2.7	**2.5**	0.24	61	39	**	

[a] Control = Millipore filtered seawater with 10% pore water added as nutrient supply for the zoospores; [b] PW = pore water only, as an additional control;

[c] ** indicates significant difference at alpha ≤0.01.

Appendix A6. Polychaete, *Dinophilus gyrociliatus*, toxicity test data for ordnance compounds: survival.

Date	Sample	Initial concentration (mg/L)	% Survival: Replicate number 1	2	3	4	5	Mean % survival	SD	Significantly different from control[b]	LC_{50} (mg/L)
10/2/98	MFS[a]	Blank control	100	100	100	100	75	**95**	11.2		
10/14/98	MFS[a]	Blank control	100	100	100	100	100	**100**	0.0		
10/14/98	2,4-DNT	38.92	0	0	0	0	0	**0**	0.0	**	20.9 (18.8-23.1)
	2,4-DNT	18.97	100	50	25	75	50	**60**	28.5	**	
	2,4-DNT	9.48	100	100	100	100	100	**100**	0.0		
	2,4-DNT	4.80	100	100	100	100	100	**100**	0.0		
	2,4-DNT	2.42	75	100	75	100	75	**85**	13.7		
10/14/98	2,6-DNT	29.60	0	0	0	0	0	**0**	0.0	**	13.2 (11.9-14.6)
	2,6-DNT	14.61	75	100	75	100	50	**80**	20.9		
	2,6-DNT	7.24	75	75	100	75	75	**80**	11.2		
	2,6-DNT	3.56	50	75	100	75	75	**75**	17.7	*	
	2,6-DNT	1.79	100	100	100	100	100	**100**	0.0		
10/2/98	2,4,6-TNT	23.63	0	0	0	0	0	**0**	0.0	**	7.70 (7.34-8.08)
	2,4,6-TNT	11.63	0	0	0	0	0	**0**	0.0	**	
	2,4,6-TNT	6.13	50	100	75	75	100	**80**	20.9		
	2,4,6-TNT	2.83	100	100	100	100	100	**100**	0.0		
	2,4,6-TNT	1.42	100	100	100	75	100	**95**	11.2		
	2,4,6-TNT	0.71	100	100	100	100	75	**95**	11.2		
	2,4,6-TNT	0.35	100	75	100	100	100	**95**	11.2		
10/2/98	RDX	48.88	100	100	100	100	100	**100**	0.0		>48.9
	RDX	23.67	75	100	100	100	100	**95**	11.2		
	RDX	11.87	100	100	50	100	100	**90**	22.4		
	RDX	6.18	100	100	50	100	100	**90**	22.4		
10/14/98	1,3-DNB	19.61	0	50	0	25	50	**25**	25.0	**	15 (14.1-16.0)
	1,3-DNB	9.74	100	100	100	100	50	**90**	22.4		
	1,3-DNB	4.45	100	25	100	100	100	**85**	33.5		
	1,3-DNB	2.42	75	100	100	50	100	**85**	22.4		
	1,3-DNB	1.20	75	100	100	100	75	**90**	13.7		

Appendix A6. Continued

Date	Sample	Initial concentration (mg/L)	% Survival: Replicate number 1	2	3	4	5	Mean % survival	SD	Significantly different from control[b]	LC_{50} (mg/L)
10/14/98	1,3,5-TNB	9.98	0	0	0	0	0	0	0.0	**	2.09 (1.93-2.26)
	1,3,5-TNB	4.90	0	0	0	0	0	0	0.0	**	
	1,3,5-TNB	2.41	50	25	25	0	75	35	28.5	**	
	1,3,5-TNB	1.18	100	75	100	75	75	85	13.7		
	1,3,5-TNB	0.61	75	100	100	100	100	95	11.2		
	1,3,5-TNB	0.35	100	100	100	100	100	100	0.0		
	1,3,5-TNB	0.13	100	100	100	100	100	100	0.0		
10/2/98	Tetryl	0.158	0	0	0	0	0	0	0.0	**	0.06 (0.05-0.07)
	Tetryl	0.056	0	25	75	100	0	40	45.4	**	
	Tetryl	0.026	100	100	100	100	100	100	0.0		
	Tetryl	0.015	100	100	75	50	75	80	20.9		
	Tetryl	0.005	100	75	100	100	100	95	11.2		
10/14/98	Picric Acid	722.45	0	0	0	0	0	0	0.0	**	264.9 95% limits not reliable
	Picric Acid	378.93	0	0	0	0	0	0	0.0	**	
	Picric Acid	198.94	50	100	100	100	100	90	22.4		
	Picric Acid	107.82	100	50	100	100	100	90	22.4		

[a]MFS: millipore filtered seawater; [b] * indicates significant difference at alpha ≤0.05, and ** indicates significant difference at alpha ≤0.01.

Appendix A7. Polychaete, *Dinophilus gyrociliatus*, toxicity test data for ordnance compounds: reproduction.

Date	Sample	Initial concentration (mg/L)	Layed Eggs/Adult Female									Significantly different from control[b]	EC_{50} (mg/L)
			Replicate number					Mean	SD	% Control	% Difference from control		
			1	2	3	4	5						
10/2/98	MFS[a]		3.5 5.83	1.75 10	8.5 6	2.5 7.67	6.67 9.67	6.21	2.9	100	0		
10/14/98	MFS[a]		3.25	6.75	5	2.25	8.75	5.20	2.6	100	0		
10/14/98	2,4-DNT	38.92	0	0	0	0	0	0.00	0.0	0	100	**	5.67 (4.99-6.43)
	2,4-DNT	18.97	0	0	0	0	0	0.00	0.0	0	100	**	
	2,4-DNT	9.48	0.25	1	1.25	1.6	0.75	0.97	0.5	19	81	**	
	2,4-DNT	4.8	3.25	2.4	5.33	2.5	2.25	3.15	1.3	60.5	39.5	*	
	2,4-DNT	2.42	1.33	4.5	3	3.25	3	3.02	1.1	58	42	*	
10/14/98	2,6-DNT	29.6	0	0	0	0	0	0.00	0.0	0	100	**	2.07 (1.39-3.09)
	2,6-DNT	14.61	0.67	0.25	0	0	0	0.18	0.3	4	96	**	
	2,6-DNT	7.24	3	0.67	2	1.67	0	1.47	1.2	28	72	**	
	2,6-DNT	3.56	1.5	2	3	1.67	2	2.03	0.6	39	61	**	
	2,6-DNT	1.79	0.75	4	3.5	2.5	3	2.75	1.3	53	47	**	
10/2/98	2,4,6-TNT	23.63	0	0	0	0	0	0.00	0.0	0	100	**	1.79 (1.57-2.05)
	2,4,6-TNT	11.63	0	0	0	0	0	0.00	0.0	0	100	**	
	2,4,6-TNT	6.13	0	0	0	0	0	0.00	0.0	0	100	**	
	2,4,6-TNT	2.83	0.75	1.25	1.75	1.75	0.5	1.20	0.6	19	81	**	
	2,4,6-TNT	1.42	3.5	5.25	3	5	4	4.15	1.0	67	33		
	2,4,6-TNT	0.71	5.2	7	5.5	6.25	6.67	6.12	0.8	99	1		
	2,4,6-TNT	0.35	2.25	3.33	8.5	3	8	5.00	2.8	80	20		
10/2/98	RDX	48.88	2	2.67	4.17	3	1.5	2.67	1.0	43	57	**	25.7 (12.7-52.2)
	RDX	23.67	1.33	3.25	4.25	2.75	3.25	2.97	1.1	48	52	**	
	RDX	11.87	6.75	3.25	5.5	7	5.8	5.66	1.5	91	9		
	RDX	6.18	9	7	6.5	4.5	5.75	6.55	1.7	105	-5		
10/14/98	1,3-DNB	19.61	0	0	0	0	0	0.00	0.0	0	100	**	3.70 (3.37-4.06)
	1,3-DNB	9.74	0	0	0	0	0	0.00	0.0	0	100	**	
	1,3-DNB	4.45	1.25	4	1.5	0.5	2.25	1.90	1.3	37	63	**	
	1,3-DNB	2.42	2.67	2	7	5.5	3.75	4.18	2.1	80	20		
	1,3-DNB	1.2	7.33	4.25	3.6	5.75	4	4.99	1.5	96	4		

Appendix A7. Continued

Date	Sample	Initial concentration (mg/L)	Layed Eggs/Adult Female									Significantly different from control[b]	EC_{50} (mg/L)
			Replicate number					Mean	SD	% Control	% Difference from control		
			1	2	3	4	5						
10/14/98	1,3,5-TNB	9.98	0	0	0	0	0	0.00	0.0	0	100	**	0.60 (0.54-0.68)
	1,3,5-TNB	4.90	0	0	0	0	0	0.00	0.0	0	100	**	
	1,3,5-TNB	2.41	0	0	0	0	0	0.00	0.0	0	100	**	
	1,3,5-TNB	1.18	0.75	0	1	0	0	0.35	0.5	7	93	**	
	1,3,5-TNB	0.61	3	3	2.75	2.5	2	2.65	0.4	51	49	**	
	1,3,5-TNB	0.35	6.75	3.75	4.75	2.75	2.8	4.16	1.7	80	20		
	1,3,5-TNB	0.13	1.8	3.4	6.5	4	4	3.94	1.7	76	24		
10/2/98	Tetryl	0.158	0	0	0	0	0	0.00	0.0	0	100	**	0.024 (0.02-0.03)
	Tetryl	0.056	0	0	1	1	0	0.40	0.5	6	94	**	
	Tetryl	0.026	3.75	5.8	3.5	2.25	3.63	3.79	1.3	61	39	*	
	Tetryl	0.015	3.5	6	3	4.5	5	4.40	1.2	71	29		
	Tetryl	0.005	4.5	9.67	2.25	6	5.25	5.53	2.7	89	11		
10/14/98	Picric Acid	722.45	0	0	0	0	0	0.00	0.0	0	100	**	154.8 (149.3-160.4)
	Picric Acid	378.93	0	0	0	0	0	0.00	0.0	0	100	**	
	Picric Acid	198.40	0.5	0.5	0.75	0	0.5	0.45	0.3	9	91	**	
	Picric Acid	107.82	5	7.5	5.25	6.75	3.25	5.55	1.7	107	-7		

[a]MFS: millipore filtered seawater; [b] * indicates significant difference at alpha ≤0.05, and ** indicates significant difference at alpha ≤0.01.

Appendix A8. Redfish, *Sciaenops ocellatus*, toxicity test survival data for ordnance compounds.

Date	Sample	Initial concentration (mg/L)	% Survival: Replicate number 1	2	3	4	5	Mean % survival	SD	Significantly different from control[b]	LC_{50} (mg/L)
9/22/98	MFS[a]	**100**	90	80	100	90	100	**90.0**	6.67		
		100	90	80	90	90	90				
10/7/98	MFS[a]	**100**	90	90	100	90	100	**94.0**	5.48		
9/22/98	2,4 DNT	**66.78**	0	0	0	0	0	**0.0**	0.00	**	**48.1 (95% limits not reliable)**
	2,4 DNT	**34.6**	100	100	90	0[c]	60[c]	**96.7**	5.77		
	2,4 DNT	**18.6**	90	80	90	90	90	**88.0**	4.47		
	2,4 DNT	**9.06**	90	90	80	80	70	**82.0**	8.37		
	2,4 DNT	**4.22**	90	80	90	80	80	**84.0**	5.48		
9/22/98	2,6 DNT	**70.11**	0	0	0	0	0	**0.0**	0.00	**	**34.2 (26.5-44.0)**
	2,6 DNT	**31.95**	60	60	40	30	40	**46.0**	13.42	**	
	2,6 DNT	**13.74**	80	80	100	100	80	**88.0**	10.95		
	2,6 DNT	**6.53**	80	90	90	80	90	**86.0**	5.48		
	2,6 DNT	**3.46**	100	100	100	90	100	**98.0**	4.47		
9/22/98	2,4,6 TNT	**27.04**	0	0	0	0	0	**0.0**	0.00	**	**8.24 (95% limits not reliable)**
	2,4,6 TNT	**10.82**	10	0	0	0	0	**2.0**	4.47	**	
	2,4,6 TNT	**6.28**	100	100	100	90	90	**96.0**	5.48		
	2,4,6 TNT	**3.08**	10[c]	90	100	90	100	**95.0**	5.77		
	2,4,6 TNT	**1.5**	100	90	90	90	100	**94.0**	5.48		
	2,4,6 TNT	**0.82**	80	100	100	90	90	**92.0**	8.37		
10/7/98	RDX	**68**	80	90	100	90	80	**88.0**	8.37		**>68.0**
	RDX	**34.6**	80	90	80	100	90	**88.0**	8.37		
	RDX	**17.2**	60[c]	90	100	100	100	**97.5**	5.00		
	RDX	**8.6**	80	70	100	90	100	**88.0**	13.04		
9/22/98	1,3 DNB	**101.57**	0	0	0	0	0	**0.0**	0.00	**	**46.0 (35.3-60.0)**
	1,3 DNB	**49.63**	30	40	50	50	90[c]	**42.5**	9.57	**	
	1,3 DNB	**25.18**	100	90	90	100	100	**96.0**	5.48		
	1,3 DNB	**13.68**	60	70	80	80	70	**72.0**	8.37	**	
	1,3 DNB	**6.27**	80	80	20[c]	80	50[c]	**80.0**	0.00		
	1,3 DNB	**3.2**	70	90	80	90	90	**84.0**	8.94		

Appendix A8. Continued.

Appendix A8. Continued.

Date	Sample	Initial concentration (mg/L)	% Survival							Significantly different from control[b]	LC_{50} (mg/L)
			Replicate number					Mean % survival	SD		
			1	2	3	4	5				
10/7/98	1,3,5 TNB	**2**	0	0	0	0	0	**0.0**	0.00	**	**1.40 (95% limits not reliable)**
	1,3,5 TNB	**0.986**	100	100	100	100	100	**100.0**	0.00		
	1,3,5 TNB	**0.476**	80	90	90	90	70	**84.0**	8.94		
	1,3,5 TNB	**0.247**	60	90	90	90	70	**80.0**	14.14		
	1,3,5 TNB	**0.123**	80	90	90	70	90	**84.0**	8.94		
	1,3,5 TNB	**0.061**	70	90	80	100	100	**88.0**	13.04		
9/22/98	Tetryl	**2.56**	0	0	0	0	0	**0.0**	0.00	**	**1.77 (95% limits not reliable)**
	Tetryl	**1.225**	90	100	100	90	100	**96.0**	5.48		
	Tetryl	**0.548**	100	100	90	100	100	**98.0**	4.47		
	Tetryl	**0.271**	90	100	90	100	90	**94.0**	5.48		
9/22/98	Picric Acid	**723**	0	0	0	0	0	**0.0**	0.00	**	**126.9 (112.8-142.8)**
	Picric Acid	**365.4**	0	0	0	0	0	**0.0**	0.00	**	
	Picric Acid	**187.2**	0	0	0	0	0	**0.0**	0.00	**	
	Picric Acid	**97.4**	70	80	70	100	90	**82.0**	13.04		
	Picric Acid	**53.9**	90	100	100	90	90	**94.0**	5.48		

[a] Millipore filetered seawater; [b] ** indicates significant difference at alpha $\leq$0.01; [c] data detected as outlier and not included in statistical analyses.

Appendix A9. Opossum shrimp, *Mysidopsis bahia,* toxicity test survival data for ordnance compounds.

Date	Sample	Initial concentration (mg/L)	% Survival: Replicate number 1	2	3	4	5	Mean % survival	SD	Significantly different from control[b]	LC_{50} (mg/L)
1/28/99	MFS[a]	100	100	80	100	100	100	93.3	8.16		
		100	100	90	100	80	90				
		100	100	90	90	100	80				
1/28/99	2,4-DNT	**14.353**	20	10	10	0	10	**10.0**	7.07	**	
	2,4-DNT	**6.839**	30	30	20	20	50[c]	**2.5**	0.58	**	**5.40 (4.21-6.92)**
	2,4-DNT	**3.581**	80	100	70	90	80	**84**	11.40		
	2,4-DNT	**1.733**	80	100	100	100	80	**92**	10.95		
	2,4-DNT	**0.878**	90	80	100	100	80	**90.0**	10.00		
1/28/99	2,6-DNT	**9.779**	10	0	0	10	0	**4**	5.48	**	
	2,6-DNT	**4.966**	90	70	60	50	70	**68**	14.83		**5.57 (4.40-7.05)**
	2,6-DNT	**2.159**	60	90	80	80	100	**82**	14.83		
	2,6-DNT	**1.144**	70	90	100	80	90	**86**	11.40		
	2,6-DNT	**0.511**	100	90	100	90	80	**92**	8.37		
1/28/99	2,4,6-TNT	**5.17**	0	0	0	0	0	**0.0**	0.00	**	
	2,4,6-TNT	**2.683**	10	0	10	0	10	**6**	5.48	**	
	2,4,6-TNT	**1.339**	40	10	10	0	40	**20.0**	18.71	**	
	2,4,6-TNT	**0.647**	60	80	80	20[c]	60	**7.0**	1.15		**0.98 (0.73-1.32)**
	2,4,6-TNT	**0.28**	100	80	90	100	80	**90.0**	10.00		
	2,4,6-TNT	**0.176**	70	40[c]	70	90	90	**8**	1.15		
	2,4,6-TNT	**0.057**	80	50[c]	90	100	90	**9**	0.82		
1/28/99	RDX	**46.699**	90	70	90	90	100	**88**	10.95		
	RDX	**22.134**	90	90	100	90	90	**92**	4.47		**>46.7**
	RDX	**11.279**	100	80	80	100	90	**90**	10.00		
	RDX	**5.663**	80	90	100	100	60[c]	**9.25**	0.96		
1/28/99	1,3-DNB	**9.70**	0.00	0.00	0.00	0.00	0.00	**0.00**	0.00	**	
	1,3-DNB	**5.19**	100.00	80.00	70.00	100.00	90.00	**88.00**	13.04		**7.10 (nr)**
	1,3-DNB	**2.48**	100.00	80.00	100.00	90.00	90.00	**92.00**	8.37		
	1,3-DNB	**1.30**	90.00	70.00	90.00	70.00	90.00	**82.00**	10.95		
	1,3-DNB	**0.71**	80.00	70.00	70.00	90.00	80.00	**78.00**	8.37		

Appendix A9. Continued

Date	Sample	Initial concentration (mg/L)	% Survival							Significantly different from control[b]	LC_{50} (mg/L)
			Replicate number					Mean % survival	SD		
			1	2	3	4	5				
1/28/99	1,3,5-TNB	**7.677**	0	0	0	0	0	**0**	0.00	**	
	1,3,5-TNB	**3.700**	0	0	0	0	0	**0**	0.00	**	**1.27 (1.04-1.55)**
	1,3,5-TNB	**1.875**	0	0	10	0	20	**6**	8.94	**	
	1,3,5-TNB	**0.961**	60	80	70	90	70	**74**	11.40		
	1,3,5-TNB	**0.540**	70	100	80	80	100	**86**	13.42		
1/28/99	Tetryl	**2.002**	0	0	0	0	0	**0**	0.00	**	
	Tetryl	**1.073**	90	40[c]	80	80	70	**8**	0.82		
	Tetryl	**0.509**	70	90	90	80	90	**84**	8.94		**1.27 (1.05-1.54)**
	Tetryl	**0.25**	90	100	90	100	80	**92**	8.37		
	Tetryl	**0.12**	100	90	100	100	90	**96**	5.48		
	Tetryl	**0.074**	80	100	90	90	80	**88**	8.37		
	Tetryl	**0.049**	100	100	100	100	100	**100**	0.00		
1/28/99	Picric Acid	**86.101**	0	0	0	0	0	**0**	0.00	**	
	Picric Acid	**42.762**	0	0	0	0	0	**0**	0.00	**	**13.0 (10.8-15.7)**
	Picric Acid	**20.557**	20	10	10	10	10	**12**	4.47	**	
	Picric Acid	**9.239**	70	70	80	80	70	**74**	5.48		
	Picric Acid	**4.832**	80	70	80	60	80	**74**	8.94		

[a]MFS: millipore filtered seawater; [b] ** indicates significant difference at alpha $\leq$0.01; [c] data detected as outlier and not included in statistical analyses.

Appendix A10. Reference toxicant (SDS) data for all tests conducted concurrently with ordnance compound toxicity tests.

Test	Date	Nominal concentration (mg/L)	% Normal: Replicate number 1	2	3	4	5	Mean % normal	SD	EC_{50} (mg/L)
Urchin fertilization	9/4/98	20	1	1	0	0	0	0.4	0.55	4.30 (3.97-4.66)
		10	1	0	1	0	0	0.4	0.55	
		5	35	39	40	23	29	33.2	7.16	
		2.5	88	83	91	89	86	87.4	3.05	
		1.25	92	94	89	91	90	91.2	1.92	
Urchin embryo dev.	9/4/98	20	0	0	0	0	0	0	0.00	3.32 (3.18-3.47)
		10	0	0	0	0	0	0	0.00	
		5	2	0	4	0	0	1.2	1.79	
		2.5	76	90	85	83	85	80	5.07	
		1.25	90	90	91	95	90	91.2	2.17	
Urchin embryo dev.	10/7/98	10	0	0	0	0	0	0	0.00	5.17 (4.76-5.62)
		5	65	51	57	33	58	52.8	12.13	
		2.5	82	93	90	89	89	80	4.04	
		1.25	80	84	87	82	86	83.8	2.86	
Urchin embryo dev.	10/19/98	10	0	0	0	0	0	80	0.00	3.76 (3.60-3.93)
		5	5	12	6	7	8	80	2.70	
		2.5	60	75	81	72	82	80	8.86	
		1.25	90	84	73	83	78	80	6.43	
Algae zoosp. germin.	1/15/99	10	0	0	0	0	0	0	0.00	2.71 (2.51-2.93)
		5	0	0	0	0	0	0	0.00	
		2.5	46	56	59	46	56	53	6.15	
		1.25	85	65	84	82	79	79	8.15	
Algae germl. length	1/15/99	10	0	0	0	0	0	0	0.00	1.65 (1.19-2.29)
		5	0	0	0	0	0	0	0.00	
		2.5	23.07	19.01	15.21	11.66	15.46	16.88	4.33	
		1.25	23.83	21.80	20.28	23.07	24.08	22.61	1.58	
Algae cell number	1/15/99	10	0.0	0.0	0.0	0.0	0.0	0.0	0.00	1.34 (0.99-1.80)
		5	0.0	0.0	0.0	0.0	0.0	0.0	0.00	
		2.5	1.9	1.2	1.0	1.0	1.2	1.3	0.37	
		1.25	1.9	2.1	2.1	2.2	2.2	2.1	0.12	

Appendix A10. Continued.

Test	Date	Nominal concentration (mg/L)	% Normal: Replicate number 1	2	3	4	5	Mean % normal	SD	EC_{50} (mg/L)
Polychaete survival	10/2/98	**10.00**	0	0	0	0	0	**0**	**0.0**	**5.95 (4.40-8.03)**
		5.00	100	100	100	25	50	**75**	**35.4**	
		2.50	100	100	100	100	100	**100**	**0.0**	
		1.25	100	100	100	100	100	**100**	**0.0**	
Polychaete survival	10/14/98	**10.00**	0	0	0	0	0	**0**	**0.0**	**4.35 (4.08-4.64)**
		5.00	50	0	25	75	0	**30**	**32.6**	
		2.50	100	100	100	100	100	**100**	**0.0**	
		1.25	100	100	100	100	100	**100**	**0.0**	
Polychaete reprod.	10/2/98	**10.00**	0	0	0	0	0	**0.00**	**0.0**	**4.18 (3.89-4.48)**
		5.00	2.33	0.8	2.5	0	3	**1.73**	**1.3**	
		2.50	5.5	4	6.4	7.25	7.4	**6.11**	**1.4**	
		1.25	7.5	5.5	6.5	6.25	3.25	**5.80**	**1.6**	
Polychaete reprod.	10/14/98	**10.00**	0	0	0	0	0	**0.00**	**0.0**	**1.98 (1.72-2.27)**
		5.00	0	0	4	1	0	**1.00**	**1.7**	
		2.50	1	2.5	1	4	0.5	**1.80**	**1.4**	
		1.25	3.8	4.5	3.25	2.75	5.25	**3.91**	**1.0**	
Red fish survival	9/22/98	**10**	0	0	0	0	0	**0.0**	**0.00**	**3.54 (95% limits not reliable)**
		5	0	0	0	0	0	**0.0**	**0.00**	
		2.5	90	100	100	100	100	**98.0**	**4.47**	
		1.25	80	100	100	90	90	**92.0**	**8.37**	
Red fish survival	10/7/98	**10**	0	0	0	0	0	**0.0**	**0.00**	**7.07 (95% limits not reliable)**
		5	90	80	80	80	100	**86.0**	**8.94**	
		2.5	100	100	100	100	100	**100.0**	**0.00**	
		1.25	100	100	100	100	100	**100.0**	**0.00**	
Mysid survival	1/28/99	**50**	0	0	0	0	0	**0**	**0.00**	**16.5 (11.9-22.8)**
		25	30	30	10	30	30	**26**	**8.94**	
		12.5	60	90	70	60	90	**74**	**15.17**	
		6.25	80	70	80	100	60	**78**	**14.83**	
		3.125	70	80	80	80	90	**80**	**7.07**	
		1.56	80	90	100	100	100	**94**	**8.94**	

Appendix B

Water quality data for toxicity tests with ordnance compounds

Appendix B1. Water quality parameters measured for stock solutions of sea urchin, *A. punctulata*, fertilization test with ordnance compounds.

Appendix B2. Water quality parameters measured for stock solutions at initiation of sea urchin, *A. punctulata*, embryological development test with ordnance compounds.

Appendix B3. Water quality parameters measured for test solutions at initiation of macro-alga, *U. fasciata*, toxicity test with ordnance compounds.

Appendix B4. Water quality parameters measured at initiation and termination of polychaete, *D. gyrociliatus*, toxicity test with ordnance compounds.

Appendix B5. Water quality parameters measured at initiation and termination of redfish, *Sciaenops ocellatus*, toxicity test with ordnance compounds.

Appendix B6. Water quality parameters measured at initiation and termination of mysid, *M. bahia*, toxicity test with ordnance compounds.

Appendix B1. Water quality parameters measured for stock solutions of sea urchin, *A. punctulata*, fertilization test with ordnance compounds.

Sample	Concentration (mg/L)	DO[a] (mg/L)	DO[a] (% sat.)	pH
MFS[b]		7.21	98.8	7.99
2,4-DNT	156.4	7.59	103.8	8.00
2,6-DNT	84.5	7.12	97.5	7.96
2,4,6-TNT	103.1	7.10	97	7.91
Picric Acid	704.4	7.36	100.2	7.89[c]
1,3-DNB	315.2	7.19	98.2	7.92
1,3,5-TNB	264.1	7.28	99.3	7.92
Tetryl	98.8	7.25	98.9	7.95
RDX	74.7	NM[d]	NM[d]	8.18

[a]Dissolved Oxygen

[b] MFS = Millipore filtered seawater;

[c]Original pH=2.80, adjusted to 7.89 for test;

[d] NM = not measured.

Appendix B2. Water quality parameters measured for stock solutions at initiation of sea urchin, *A. punctulata*, embryological development test with ordnance compounds.

Sample	Concentration (mg/L)	DO[a] (mg/L)	DO[a] (% sat.)	pH
MFS[a]		7.21	98.8	7.99
2,4-DNT	156.4	7.59	103.8	8.00
2,6-DNT	86.4	7.44	101.4	7.90
2,4,6-TNT	79.9	7.15	97.0	7.95
Picric Acid	704.4	7.36	100.2	7.89[c]
1,3-DNB	315.2	7.19	98.2	7.92
1,3,5-TNB	2.0	6.87	93.9	7.92
Tetryl	0.2	6.75	91.8	7.97
RDX	74.7	NM[d]	NM[d]	8.18

[a]Dissolved Oxygen

[b]MFS = Millipore filtered seawater;

[c]Original pH=2.80, adjusted to 7.89 at test start; pH measured again at test end and had dropped to 6.67 at highest test concentration, but was between 7.37 and 7.56 in subsequent 4 test concentrations;

[d]NM = not measured.

Appendix B3. Water quality parameters measured for test solutions at initiation of macro-alga, *U. fasciata*, toxicity test with ordnance compounds.

Sample	Conc. (mg/L)	DO (mg/L)	DO (% sat.)	pH
Control	100.0	7.16	97.5	8.00
2,4-DNT	3.5	7.53	101.3	7.89
2,4-DNT	1.8	7.57	102.7	7.89
2,4-DNT	0.9	7.64	102.8	7.91
2,4-DNT	0.5	7.49	101.3	7.91
2,6-DNT	19.7	7.53	101.7	7.88
2,6-DNT	9.5	7.44	100.2	7.91
2,6-DNT	4.7	7.53	101.1	7.91
2,6-DNT	2.2	7.50	101.3	7.91
2,6-DNT	1.2	7.68	103.3	7.92
2,4,6-TNT	3.4	7.49	101.2	7.92
2,4,6-TNT	1.7	7.50	101.4	7.92
2,4,6-TNT	0.9	7.57	101.8	7.90
2,4,6-TNT	0.4	7.56	102.1	7.91
2,4,6-TNT	0.2	7.59	102.3	7.92
RDX	31.3	6.91	93.2	7.83
RDX	15.7	6.93	93.1	7.88
RDX	9.2	6.92	93.3	7.91
RDX	5.0	6.78	92.0	7.85
1,3-DNB	2.5	6.92	93.8	7.93
1,3-DNB	1.2	6.90	93.4	7.91
1,3-DNB	0.6	6.91	93.6	7.92
1,3-DNB	0.3	6.98	94.8	7.93
1,3-DNB	0.2	6.99	94.5	7.95
1,3,5-TNB	0.2	7.30	99.0	7.94
1,3,5-TNB	0.1	7.19	97.2	7.94
1,3,5-TNB	0.1	7.31	98.7	7.93
1,3,5-TNB	0.0	7.25	98.2	7.93
Tetryl	1.0	6.84	92.5	7.93
Tetryl	0.5	7.03	95.0	7.91
Tetryl	0.3	6.95	93.9	7.94
Tetryl	0.1	6.93	93.8	7.92
Picric Acid	662.7	7.57	102.3	7.95
Picric Acid	336.1	7.51	105.5	7.94
Picric Acid	169.2	7.42	100.4	7.93
Picric Acid	92.1	7.50	101.5	7.91

Appendix B4. Water quality parameters measured at initiation and termination of polychaete, *D. gyrociliatus*, toxicity test with ordnance compounds.

Test Date	Chemical	Conc. mg/L	Test Start DO mg/L	Test Start DO % Satur.	Test Start pH	Test End DO mg/L	Test End DO % Satur.	Test End pH
10/2/98	MFS		7.29	99.4	7.90	7.35	101.4	7.81
10/14/98	MFS		6.94	95.0	7.99	7.45	102.5	7.79
10/14/98	2,4-DNT	38.9	8.51	116.9	7.90	6.94	95.3	7.82
	2,4-DNT	19.0	8.54	117.6	7.93	6.97	95.6	7.81
	2,4-DNT	9.5	8.46	116.5	7.95	6.96	95.5	7.81
	2,4-DNT	4.8	8.44	116.4	7.96	7.05	96.1	7.84
	2,4-DNT	2.4	8.38	115.5	7.95	7.03	95.9	7.86
10/14/98	2,6-DNT	29.6	8.53	116.9	8.05	7.48	102.5	7.82
	2,6-DNT	14.6	8.54	117.4	8.01	7.38	101.4	7.80
	2,6-DNT	7.2	8.56	117.6	7.97	7.45	102.6	7.79
	2,6-DNT	3.6	8.47	116.4	7.96	7.42	102.3	7.81
	2,6-DNT	1.8	8.53	117.3	7.96	7.43	102.6	7.81
10/2/98	2,4,6-TNT	23.6	8.01	108.9	7.87	7.50	102.0	7.84
	2,4,6-TNT	11.6	7.50	101.7	7.89	7.53	102.5	7.85
	2,4,6-TNT	6.1	7.27	98.6	7.90	7.42	101.4	7.86
	2,4,6-TNT	2.8	7.11	96.5	7.90	7.37	101.0	7.78
	2,4,6-TNT	1.4	7.16	97.2	7.90	7.43	101.6	7.80
	2,4,6-TNT	0.7	7.27	98.7	7.90	7.29	100.9	7.79
	2,4,6-TNT	0.4	7.33	99.4	7.90	7.33	100.7	7.79
10/2/98	RDX	48.9	7.75	105.3	8.00	7.31	99.7	7.81
	RDX	23.7	7.60	103.3	7.92	7.51	102.1	7.82
	RDX	11.9	7.53	102.3	7.90	7.57	102.4	7.82
	RDX	6.2	7.48	101.8	7.90	7.52	101.9	7.83
10/14/98	1,3-DNB	19.6	8.71	119.1	7.95	6.90	94.8	7.82
	1,3-DNB	9.7	8.68	118.8	7.94	6.99	96.1	7.83
	1,3-DNB	4.5	8.59	117.8	7.95	6.86	94.7	7.82
	1,3-DNB	2.4	8.60	117.9	7.95	6.90	95.3	7.78
	1,3-DNB	1.2	8.58	117.8	7.95	6.91	95.7	7.78
10/14/98	1,3,5-TNB	10.0	6.85	94.0	7.97	7.46	102.6	7.82
	1,3,5-TNB	4.9	6.83	93.8	7.97	7.41	101.8	7.79
	1,3,5-TNB	2.4	6.85	94.1	7.97	7.46	102.6	7.77
	1,3,5-TNB	1.2	6.85	94.1	7.97	7.43	102.1	7.77
	1,3,5-TNB	0.6	6.80	93.3	7.97	7.41	102.1	7.80
	1,3,5-TNB	0.4	6.92	95.1	7.98	7.37	101.8	7.79
	1,3,5-TNB	0.1	6.81	93.6	7.98	7.37	101.4	7.77
10/2/98	Tetryl	0.2	7.18	98.2	7.91	7.48	102.2	7.84
	Tetryl	0.1	7.16	97.8	7.91	7.44	101.7	7.81
	Tetryl	0.0	7.15	97.8	7.91	7.42	101.4	7.82
	Tetryl	0.0	7.18	98.0	7.91	7.39	101.2	7.82
	Tetryl	0.0	7.26	99.0	7.91	7.38	101.8	7.81
10/14/98	Picric Acid	722.5	8.40	116.2	8.16	7.45	102.2	7.18
	Picric Acid	378.9	8.80	119.9	8.01	7.44	102.1	7.59
	Picric Acid	198.9	8.56	116.9	7.98	7.37	101.3	7.67
	Picric Acid	107.8	8.40	115.1	7.96	7.38	101.5	7.74

Appendix B5. Water quality parameters measured at initiation and termination of redfish, *Sciaenops ocellatus*, toxicity test with ordnance compounds.

Date	Sample	Conc. (mg/L)	Test Start			Test End		
			DO (mg/L)	DO (% sat.)	pH	DO (mg/L)	DO (% sat.)	pH
9/22/98	MFS		6.12	84.0	8.01	6.73	93.8	7.86
10/7/98	MFS		6.83	91.2	7.98	6.58	91.6	7.90
9/22/98	2,4-DNT	66.8	6.78	93.2	7.97	6.96	96.6	7.85
	2,4-DNT	34.6	6.28	86.3	7.96	6.96	96.8	7.86
	2,4-DNT	18.6	6.25	85.7	8.01	6.88	95.6	7.85
	2,4-DNT	9.1	6.25	85.5	8.00	6.82	95.1	7.82
	2,4-DNT	4.2	6.23	85.1	8.03	6.74	94.2	7.84
9/22/98	2,6-DNT	70.1	6.92	94.8	7.91	6.38	88.1	7.77
	2,6-DNT	32.0	6.43	88.1	7.96	6.36	87.7	7.79
	2,6-DNT	13.7	6.25	85.7	7.98	6.57	90.8	7.86
	2,6-DNT	6.5	6.18	84.6	8.02	6.41	88.7	7.83
	2,6-DNT	3.5	6.19	84.9	7.98	6.62	91.9	7.86
9/22/98	2,4,6-TNT	27.0	6.51	89.0	7.99	6.52	90.2	7.74
	2,4,6-TNT	10.8	6.18	84.7	8.00	6.34	87.7	7.80
	2,4,6-TNT	6.3	6.23	85.2	8.02	6.68	92.4	7.85
	2,4,6-TNT	3.1	6.12	83.7	8.00	6.53	90.4	7.84
	2,4,6-TNT	1.5	6.14	83.9	8.02	6.58	91.2	7.84
	2,4,6-TNT	0.8	6.12	83.9	8.02	6.67	92.6	7.84
10/7/98	RDX	68.0	6.28	84.3	7.93	6.59	92.3	7.78
	RDX	34.6	6.65	88.7	7.94	6.57	92.7	7.85
	RDX	17.2	6.70	89.2	7.94	6.63	91.7	7.89
	RDX	8.6	6.68	89.0	7.94	6.64	92.1	7.89
9/22/98	1,3-DNB	101.6	7.01	96.7	8.24	6.79	94.1	7.86
	1,3-DNB	49.6	6.41	87.4	8.11	6.57	91.1	7.86
	1,3-DNB	25.2	6.24	85.3	8.06	6.57	91.7	7.87
	1,3-DNB	13.7	6.24	85.2	8.01	6.55	91.9	7.84
	1,3-DNB	6.3	6.19	84.6	8.05	6.45	89.8	7.84
	1,3-DNB	3.2	6.22	85.1	7.99	6.39	89.1	7.82
10/7/98	1,3,5-TNB	2.0	8.97	121.3	7.83	6.77	94.7	7.67
	1,3,5-TNB	1.0	8.91	120.2	7.83	6.57	91.5	7.87
	1,3,5-TNB	0.5	8.90	119.8	7.84	6.56	91.4	7.84
	1,3,5-TNB	0.3	8.93	120.0	7.84	6.44	90.6	7.83
	1,3,5-TNB	0.1	8.93	119.9	7.84	6.56	92.6	7.82
	1,3,5-TNB	0.1	8.93	120.0	7.81	6.56	92.4	7.82
9/22/98	Tetryl	2.6	6.36	86.2	7.97	6.55	91.3	7.80
	Tetryl	1.2	6.23	84.6	7.99	6.74	92.7	7.85
	Tetryl	0.5	6.21	84.7	7.99	6.78	93.8	7.84
	Tetryl	0.3	6.23	85.2	7.95	6.55	90.7	7.86
9/22/98	Picric Acid	723.0	6.55	89.3	8.08	6.63	91.9	6.99
	Picric Acid	365.4	6.29	85.8	8.00	6.45	89.4	7.56
	Picric Acid	187.2	6.34	86.5	8.02	6.38	88.3	7.67
	Picric Acid	97.4	6.20	85.0	8.01	6.55	90.8	7.78
	Picric Acid	53.9	6.08	84.4	8.05	6.55	91.3	7.83

Appendix B6. Water quality parameters measured at initiation and termination of opossum shrimp, *M. bahia*, toxicity test with ordnance compounds.

Sample	Conc. (mg/L)	Test Start			Test End				
		DO (mg/L)	DO (% sat.)	pH	DO (mg/L)	DO (% sat.)	pH	NH_4 (mg/L)	NH_3 (ug/L)
MFS		7.04	97.7	8.02	6.15	84.8	7.61	0.583	7.59
2,4-DNT	14.4	6.68	92.5	7.97	5.99	82.5	7.50	0.563	5.70
2,4-DNT	6.8	6.59	91.8	7.97	5.70	78.6	7.37	1.050	7.91
2,4-DNT	3.6	6.49	90.6	7.97	5.46	75.5	7.41	0.040	0.33
2,4-DNT	1.7	6.41	89.9	7.98	5.63	77.9	7.34	1.020	7.17
2,4-DNT	0.9	6.74	94.0	7.96	6.24	86.8	7.49	1.030	10.20
2,6-DNT	9.8	6.55	91.9	7.99	6.09	84.5	7.59	0.849	10.56
2,6-DNT	5.0	6.61	91.9	7.99	5.70	79.1	7.48	1.420	13.74
2,6-DNT	2.2	6.64	92.2	8.00	5.80	80.1	7.51	1.320	13.68
2,6-DNT	1.1	6.53	90.9	7.99	5.94	82.2	7.49	1.230	12.18
2,6-DNT	0.5	6.59	92.0	7.99	5.73	79.3	7.42	1.250	10.55
2,4,6-TNT	5.2	6.53	91.0	8.01	NM[a]	NM	NM	NM	NM
2,4,6-TNT	2.7	6.55	91.0	8.01	6.30	87.2	7.65	0.831	11.84
2,4,6-TNT	1.3	6.17	86.0	7.98	6.08	84.1	7.61	0.952	12.39
2,4,6-TNT	0.6	6.28	87.1	8.01	5.69	78.7	7.44	0.992	8.76
2,4,6-TNT	0.3	6.40	88.9	8.00	5.94	81.9	7.56	0.906	10.52
2,4,6-TNT	0.2	6.52	90.5	8.00	5.96	82.4	7.51	0.870	9.02
2,4,6-TNT	0.1	6.53	90.9	8.00	5.79	80.0	7.43	0.838	7.24
RDX	46.7	7.04	97.4	8.05	6.20	85.7	7.58	0.573	6.96
RDX	22.1	7.08	97.9	8.02	6.21	85.9	7.57	0.599	7.12
RDX	11.3	6.95	96.8	8.00	6.17	85.4	7.57	0.633	7.52
RDX	5.7	6.97	97.0	8.01	5.90	81.5	7.60	0.651	8.28
1,3-DNB	9.7	6.40	88.9	7.99	6.15	85.0	7.50	0.699	7.08
1,3-DNB	5.2	6.31	87.7	7.97	6.04	83.6	7.52	0.794	8.42
1,3-DNB	2.5	6.40	89.0	7.99	5.32	73.8	7.40	0.785	6.33
1,3-DNB	1.3	6.37	88.7	7.98	5.65	78.2	7.42	0.877	7.40
1,3-DNB	0.7	6.56	91.2	7.96	5.68	78.8	7.49	0.772	7.64
1,3,5-TNB	7.7	6.35	87.8	8.00	NM	NM	NM	NM	NM
1,3,5-TNB	3.7	6.40	88.5	8.00	NM	NM	NM	NM	NM
1,3,5-TNB	1.9	6.47	89.6	8.00	6.20	85.6	7.56	0.671	7.79
1,3,5-TNB	1.0	6.36	88.2	8.00	5.70	78.8	7.52	0.778	8.25
1,3,5-TNB	0.5	6.41	88.9	8.01	5.96	82.5	7.53	0.728	7.90
Tetryl	2.0	6.37	88.4	7.99	NM	NM	NM	NM	NM
Tetryl	1.1	6.37	88.6	7.99	6.22	86.1	7.64	0.580	8.08
Tetryl	0.5	6.36	87.8	7.99	5.49	76.0	7.54	0.582	6.46
Tetryl	0.2	6.33	88.2	8.00	5.67	78.5	7.58	0.609	7.40
Tetryl	0.1	6.43	89.3	7.99	5.65	78.2	7.51	0.602	6.24
Tetryl	0.1	6.40	89.0	7.98	6.03	83.5	7.70	0.577	9.21
Tetryl	0.1	6.47	89.7	7.99	6.02	83.3	7.62	0.568	7.56
Picric Acid	86.1	6.48	89.6	7.66	NM+F5	NM	NM	NM	NM
Picric Acid	42.8	6.32	87.0	7.86	NM	NM	NM	NM	NM
Picric Acid	20.6	6.65	91.9	7.95	6.08	83.8	7.57	0.503	5.98
Picric Acid	9.2	6.48	89.6	7.98	5.18	71.4	7.37	0.702	5.29
Picric Acid	4.8	6.43	89.0	7.99	5.57	76.7	7.27	0.762	4.56

[a] NM = not measured

Appendix C

Measured concentrations of test solutions at initiation and termination of toxicity tests with ordnance compounds

Appendix C1. Measured concentrations of ordnance compounds in test solutions at initiation and termination of sea urchin, *A. punctulata*, embryological development test.

Appendix C2. Measured concentrations of ordnance compounds in test solutions at initiation and termination of macro-alga, *U. fasciata*, zoospore germination test. Includes vials to which no zoospores were added, in selected concentrations.

Appendix C3. Measured concentrations of ordnance compounds in test solutions at initiation and termination of polychaete, *D. gyrociliatus*, toxicity test.

Appendix C4. Measured concentrations of ordnance compounds in test solutions at initiation and termination of redfish, *S. ocellatus*, toxicity test.

Appendix C5. Measured concentrations of ordnance compounds in test solutions at initiation and termination of mysid, *M. bahia*, toxicity test.

Appendix C1. Measured concentrations of ordnance compounds in test solutions at initiation and termination of sea urchin, *A. punctulata*, embryological development test. Percent of initial represents the degradation of the chemical during the 48 hours of test duration: values >100 are caused by the chemical method intrinsic variability.

Sample	Measured concentration (mg/L)		% of Initial
	Test Start	Test End	
2,4-DNT	75.2	85.5	114
2,4-DNT	38.6	41.0	106
2,4-DNT	18.4	19.8	108
2,4-DNT	8.9	9.9	111
2,6-DNT	86.4	93.8	109
2,6-DNT	42.6	44.8	105
2,6-DNT	21.8	21.3	98
2,6-DNT	10.3	10.6	103
2,6-DNT	5.0	5.5	110
2,4,6-TNT	79.0	78.2	99
2,4,6-TNT	39.2	43.9	112
2,4,6-TNT	19.1	21.2	111
2,4,6-TNT	9.1	9.7	107
2,4,6-TNT	2.1	2.5	119
RDX	74.7	NM[a]	
RDX	26.4	NM[a]	
1,3-DNB	315.2	293.2	93
1,3-DNB	109.8	109.5	100
1,3-DNB	84.3	84.9	101
1,3,5-TNB	2.020	2.196	109
1,3,5-TNB	1.090	1.093	100
1,3,5-TNB	0.476	0.529	111
1,3,5-TNB	0.240	0.253	105
1,3,5-TNB	0.120	0.109	91
Tetryl	0.162	0.138	85
Tetryl	0.083	0.027	33
Tetryl	0.036	0.007	19
Tetryl	0.014	BDL[b]	0
Picric Acid	704.4	726.0	103
Picric Acid	352.3	423.0	120
Picric Acid	177.6	177.6	100

[a]NM = not measured

[b]BDL= below detection limits

Appendix C2. Measured concentrations of ordnance compounds in test solutions at initiation and termination of macro-alga, *U. fasciata*, zoospore germination test. Included vials to which no zoospores were added, in selected concentrations. Percent of initial represents the percentage of the chemical measured at the end of the test relative to the beginning.

Sample	Measured concentration (mg/L)		% of Initial
	Test Start	Test End	
2,4-DNT	3.47	3.70	106.6
2,4-DNT	1.77	1.66	93.8
2,4-DNT	0.94	0.83	88.3
2,4-DNT	0.48	0.43	89.6
2,6-DNT	19.7	18.1	91.9
2,6-DNT	9.5	8.9	93.7
2,6-DNT	4.7	4.2	89.4
2,6-DNT	2.2	2.0	90.9
2,6-DNT	1.2	0.9	75.0
2,4,6-TNT	3.44	2.33	67.7
2,4,6-TNT	1.74	1.16	66.7
2,4,6-TNT	0.90	0.52	57.8
2,4,6-TNT	0.43	0.22	51.2
2,4,6-TNT	0.21	0.16	76.2
RDX	31.3	41.9	133.9
RDX	15.7	20.4	129.9
RDX	9.2	10.1	109.8
RDX	5.0	4.9	98.0
1,3-DNB	2.51	2.42	96.4
1,3-DNB	1.26	1.24	98.4
1,3-DNB	0.65	0.58	89.2
1,3-DNB	0.31	0.33	106.5
1,3-DNB	0.21	0.15	71.4
1,3,5-TNB	0.186	0.033	17.7
1,3,5-TNB	0.093	0.012	12.9
1,3,5-TNB	0.046	0.004	8.7
1,3,5-TNB	0.029	0.000	0.0
Tetryl	1.003	0.344	34.3
Tetryl	0.503	0.109	21.7
Tetryl	0.250	0.015	6.0
Tetryl	0.098	0.003	3.1
Picric Acid	662.7	661.9	99.9
Picric Acid	336.1	335.1	99.7
Picric Acid	169.2	173.9	102.8
Picric Acid	92.1	84.6	91.9

Appendix C2. Continued.

No zoospores added to test vial			
Sample	Measured concentration (mg/L)		% of Initial
	Test Start	Test End	
2,4-DNT	3.474	3.443	99.1
2,6-DNT	19.738	19.108	96.8
2,4,6-TNT	3.438	2.797	81.4
RDX	15.689	20.762	132.3
1,3-DNB	2.515	2.610	103.8
1,3,5-TNB	0.186	0.046	24.7
Tetryl	1.003	0.328	32.7
Picric Acid	92.090	91.310	99.2

Appendix C3. Measured concentrations of ordnance compounds in test solutions at initiation and termination of polychaete, *D. gyrociliatus*, toxicity test. Percent of initial represents the percentage of the chemical measured at the end of the test relative to the beginning.

Sample	Measured concentration (mg/L)		% of Initial
	Test Start	Test End	
2,4-DNT	38.9	36.8	94.6
2,4-DNT	19.0	17.7	93.2
2,4-DNT	9.5	8.6	90.5
2,4-DNT	4.8	4.0	83.3
2,4-DNT	2.4	1.9	79.2
2,6-DNT	29.6	26.8	90.5
2,6-DNT	14.6	13.1	89.7
2,6-DNT	7.2	6.2	86.4
2,6-DNT	3.6	3.0	83.3
2,6-DNT	1.8	1.4	77.8
2,4,6-TNT	23.60	18.80	79.7
2,4,6-TNT	11.60	7.60	65.5
2,4,6-TNT	6.10	2.50	41.0
2,4,6-TNT	2.80	0.81	28.9
2,4,6-TNT	1.40	0.06	4.3
2,4,6-TNT	0.71	0.00	0.0
2,4,6-TNT	0.35	0.00	0.0
RDX	48.9	47.9	98.0
RDX	23.7	25.2	106.3
RDX	11.9	11.9	100.0
RDX	6.2	6.1	98.4
1,3-DNB	19.60	17.88	91.2
1,3-DNB	9.70	8.55	88.1
1,3-DNB	4.40	4.26	96.8
1,3-DNB	2.40	1.83	76.3
1,3-DNB	1.20	0.71	59.2
1,3,5-TNB	10.00	8.04	80.4
1,3,5-TNB	4.90	3.62	73.9
1,3,5-TNB	2.40	1.55	64.6
1,3,5-TNB	1.20	1.08	90.0
1,3,5-TNB	0.61	0.11	18.0
1,3,5-TNB	0.34	0.06	17.6
1,3,5-TNB	0.13	0.00	0.0
Tetryl	0.158	0	0.0
Tetryl	0.056	0	0.0
Tetryl	0.026	0	0.0
Tetryl	0.015	0	0.0
Tetryl	0.005	0	0.0
Picric Acid	722.4	710.8	98.4
Picric Acid	378.9	383.6	101.2
Picric Acid	198.9	211.2	106.2
Picric Acid	107.8	112.2	104.0

Appendix C4. Measured concentrations of ordnance compounds in test solutions at initiation and termination of redfish, *S. ocellatus*, toxicity test. Percent of initial represents the percentage of the chemical measured at the end of the test relativeto the beginning.

Sample	Measured Concentration (mg/L)		% of Initial
	Test start	Test end	
2,4-DNT	66.8	72.6	108.6
2,4-DNT	34.6	36.6	105.9
2,4-DNT	18.6	17.7	95.1
2,4-DNT	9.1	NM[a]	
2,4-DNT	4.2	4.1	96.2
2,6-DNT	70.1	51.7	73.8
2,6-DNT	32.0	29.5	92.5
2,6-DNT	13.7	12.6	91.5
2,6-DNT	6.5	6.1	92.8
2,6-DNT	3.5	3.1	90.7
2,4,6-TNT	27.0	15.1	55.9
2,4,6-TNT	10.8	9.8	90.4
2,4,6-TNT	6.3	4.5	71.6
2,4,6-TNT	3.1	1.7	56.0
2,4,6-TNT	1.5	0.6	41.1
2,4,6-TNT	0.8	NM[a]	
RDX	68.0	51.3	75.5
RDX	34.6	33.1	95.9
RDX	17.2	16.7	96.9
RDX	8.6	8.4	98.0
1,3-DNB	101.6	104.7	103.1
1,3-DNB	49.6	50.5	101.8
1,3-DNB	25.2	25.0	99.3
1,3-DNB	13.7	12.1	88.2
1,3-DNB	6.3	5.7	90.8
1,3-DNB	3.2	2.8	86.7
1,3,5-TNB	2.000	1.624	81.2
1,3,5-TNB	0.986	0.739	74.9
1,3,5-TNB	0.476	0.221	46.4
1,3,5-TNB	0.247	0.068	27.5
1,3,5-TNB	0.123	0.024	19.5
1,3,5-TNB	0.061	0.008	13.1
Tetryl	2.563	0.633	24.7
Tetryl	1.225	0.349	28.5
Tetryl	0.548	0.159	29.0
Tetryl	0.271	0.073	26.9
Picric Acid	723.0	762.5	105.5
Picric Acid	365.4	448.5	122.7
Picric Acid	187.2	201.1	107.4
Picric Acid	97.4	109.9	112.8
Picric Acid	53.9	58.8	109.0

[a] NM = not measured

Appendix C5. Measured concentrations of ordnance compounds in test solutions at initiation and termination of oposum shrimp *M. bahia*, toxicity test. Percent of initial represents the percentage of the chemical measured at the end of the test relative to the beginning.

Chemical	Measured concentration (mg/L)		% of Initial
	Initial	Final	
2,4-DNT	14.35	12.260	85.4
2,4-DNT	6.84	6.180	90.4
2,4-DNT	3.58	2.730	76.3
2,4-DNT	1.73	1.230	71.1
2,4-DNT	0.88	0.570	64.8
2,6-DNT	9.78	9.380	95.9
2,6-DNT	4.97	4.130	83.1
2,6-DNT	2.16	2.105	97.5
2,6-DNT	1.14	0.860	75.4
2,6-DNT	0.51	0.400	78.4
2,4,6-TNT	5.17	NM[a]	1.6
2,4,6-TNT	2.68	0.082	3.5
2,4,6-TNT	1.34	0.095	0.0
2,4,6-TNT	0.65	0.000	0.0
2,4,6-TNT	0.28	0.000	0.0
2,4,6-TNT	0.18	0.000	0.0
2,4,6-TNT	0.06	0.000	
RDX	46.7	46.400	99.4
RDX	22.1	21.400	96.8
RDX	11.3	11.000	97.3
RDX	5.7	5.500	96.5
1,3-DNB	9.7	8.380	86.4
1,3-DNB	5.2	3.600	69.4
1,3-DNB	2.5	1.540	62.1
1,3-DNB	1.3	0.580	44.6
1,3-DNB	0.7	0.290	40.8
1,3,5-TNB	7.68	NM	
1,3,5-TNB	3.70	NM	
1,3,5-TNB	1.88	0.557	29.6
1,3,5-TNB	0.96	0.171	17.8
1,3,5-TNB	0.54	0.031	5.7
Tetryl	2.00	0.000	-0.0
Tetryl	1.07	0.004	-0.4
Tetryl	0.51	0.001	0.2
Tetryl	0.25	0.002	0.8
Tetryl	0.12	0.002	1.7
Tetryl	0.07	0.000	0.0
Tetryl	0.05	0.000	0.0
Picric Acid	86.1	NM[a]	
Picric Acid	42.8	NM	
Picric Acid	20.6	19.058	92.5
Picric Acid	9.2	7.300	79.3
Picric Acid	4.8	4.100	85.4

[a] Not measured

Appendix D

Complete data set for toxicity tests with pore water from Puget Sound

Appendix D1. Toxicity data for sea urchin, *Arbacia punctulata*, fertilization test with pore water from 55 stations at Jackson Park and Port Hadlock sites, Puget Sound.

Appendix D2. Toxicity data for sea urchin, *Arbacia punctulata*, embryological development test with pore water from 55 stations at Jackson Park and Port Hadlock sites, Puget Sound.

Appendix D3. Water quality measurements for pore water from 55 stations at Jackson Park and Port Hadlock sites, Puget Sound, used in urchin fertilization and embryological development tests.

Appendix D4. Concentrations of a wide range of chemicals measured in selected sediment samples from Jackson Park and Port Hadlock sites, Puget Sound.

Appendix D5. Grains size distribution in selected sediment samples from Jackson Park and Port Hadlock sites, Puget Sound.

Appendix D1. Toxicity data for sea urchin, *Arbacia punctulata*, fertilization test with 55 porewater samples from stations at Jackson Park and Port Hadlock sites, and Sequim Bay, Puget Sound.

Station	Dilution (%)	% Fertilized: Replicate number 1	2	3	4	5	Mean % fertilized	SD	Significantly different from control[e]
REF[a]	100	96	97	95	93	94	93.8	2.10	
REF	100	90	92	95	92	94			
REF	50	97	95	99	97	97	96.7	1.42	
REF	50	97	94	97	98	96			
REF	25	92	95	95	96	91	95.5	2.64	
REF	25	95	96	100	97	98			
OB1	100	94	96	96	94	97	95.4	1.34	
OB1	50	96	94	96	90	99	95.0	3.32	
OB1	25	96	98	92	92	97	95.0	2.83	
OB2	100	94	98	96	95	99	96.4	2.07	
OB2	50	92	96	93	98	95	94.8	2.39	
OB2	25	96	97	40[d]	96	95	96.0	0.82	
OB3	100	78	87	84	70	78	79.4	6.54	
OB3	50	94	99	95	95	98	96.2	2.17	
OB3	25	96	92	94	98	96	95.2	2.28	
OB4	100	92	93	95	90	93	92.6	1.82	
OB4	50	95	98	91	95	96	95.0	2.55	
OB4	25	96	94	95	95	97	95.4	1.14	
OB5	100	54	40	39	42	38	42.6	6.54	**
OB5	50	91	84	88	89	90	88.4	2.70	
OB5	25	98	98	91	96	99	96.4	3.21	
OB6	100	98	95	93	94	97	95.4	2.07	
OB6	50	97	97	98	96	96	96.8	0.84	
OB6	25	98	97	98	97	94	96.8	1.64	
OB7	100	45	54	53	61	56	53.8	5.81	**
OB7	50	94	93	90	89	93	91.8	2.17	
OB7	25	95	97	94	94	91	94.2	2.17	

Appendix D1. Continued.

Station	Dilution (%)	% Fertilized							Significantly different from control[e]
		Replicate number					Mean		
		1	2	3	4	5	% fertilized	SD	
OB8	**100**	12	7	6	6	3	**6.8**	3.27	**
OB8	**50**	63	42	54	56	62	**55.4**	8.41	**
OB8	**25**	89	89	89	93	92	**90.4**	1.95	
OB9	**100**	97	92	98	96	96	**95.8**	2.28	
OB9	**50**	95	99	95	94	98	**96.2**	2.17	
OB9	**25**	94	92	97	99	98	**96.0**	2.92	
OB10	**100**	95	88	92	94	88	**91.4**	3.29	
OB10	**50**	98	95	94	96	99	**96.4**	2.07	
OB10	**25**	87	93	97	98	91	**93.2**	4.49	
OB11	**100**	26	18	13	21	10	**17.6**	6.35	**
OB11	**50**	82	76	83	73	76	**78.0**	4.30	**
OB11	**25**	93	98	97	93	92	**94.6**	2.70	
OB12	**100**	2	1	2	4	2	**2.2**	1.10	**
OB12	**50**	3	9	12	8	18	**10.0**	5.52	**
OB12	**25**	36	20	25	21	22	**24.8**	6.53	**
OB13	**100**	90	95	96	97	99	**95.4**	3.36	
OB13	**50**	95	95	97	94	94	**95.0**	1.22	
OB13	**25**	94	96	95	99	99	**96.6**	2.30	
OB14	**100**	56	62	65	47	55	**57.0**	6.96	**
OB14	**50**	90	94	85	96	93	**91.6**	4.28	
OB14	**25**	94	97	98	95	93	**95.4**	2.07	
OB15	**100**	13	10	18	10	16	**13.4**	3.58	**
OB15	**50**	74	80	79	75	81	**77.8**	3.11	**
OB15	**25**	94	97	97	92	94	**94.8**	2.17	
OB16	**100**	6	9	6	11	18	**10.0**	4.95	**
OB16	**50**	29	39	33	45	39	**37.0**	6.16	**
OB16	**25**	67	63	82	63	76	**70.2**	8.47	**
OB17	**100**	30	39	43	49	37	**39.6**	7.06	**
OB17	**50**	78	82	79	68	81	**77.6**	5.59	**
OB17	**25**	95	93	94	93	99	**94.8**	2.49	

Appendix D1. Continued.

Station	Dilution (%)	% Fertilized							Significantly different from control[e]
		Replicate number					Mean		
		1	2	3	4	5	% fertilized	SD	
OB18	**100**	12	14	10	9	13	**11.6**	2.07	**
OB18	**50**	80[d]	57[d]	28	31	43	**34.0**	7.94	**
OB18	**25**	93	86	86	88	87	**88.0**	2.92	
OB19	**100**	97	93	90	92	96	**93.6**	2.88	
OB19	**50**	97	95	96	95	99	**96.4**	1.67	
OB19	**25**	92	94	96	97	97	**95.2**	2.17	
OB20	**100**	35	41	29	30	23	**31.6**	6.77	**
OB20	**50**	94	90	91	89	92	**91.2**	1.92	
OB20	**25**	95	97	96	96	94	**95.6**	1.14	
OB21	**100**	97	99	94	99	95	**96.8**	2.28	
OB21	**50**	94	98	96	95	98	**96.2**	1.79	
OB21	**25**	93	96	94	94	98	**95.0**	2.00	
OB22	**100**	62	66	70	52	65	**63.0**	6.78	**
OB22	**50**	87	86	74[d]	88	92	**88.2**	2.63	
OB22	**25**	97	91	96	94	93	**94.2**	2.39	
OB23	**100**	43	14[d]	35	32	35	**36.2**	4.72	**
OB23	**50**	50	71[d]	46	35	41	**43.0**	6.48	**
OB23	**25**	86	88	65	71	80	**78.0**	9.82	*
OB24	**100**	38	64[d]	39	51	44	**43.0**	5.94	**
OB24	**50**	90	88	90	88	90	**89.2**	1.10	
OB24	**25**	96	87	97	90	97	**93.4**	4.62	
OB25	**100**	93	97	94	96	97	**95.4**	1.82	
OB25	**50**	95	96	94	95	95	**95.0**	0.71	
OB25	**25**	94	96	100	97	97	**96.8**	2.17	
OB26 (rep OB8)	**100**	18	28	17	14	30	**21.4**	7.13	**
OB26 (rep OB8)	**50**	59	42	59	49	63	**54.4**	8.65	**
OB26 (rep OB8)	**25**	92	85	83	87	83	**86.0**	3.74	

Appendix D1. Continued.

Station	Dilution (%)	% Fertilized: Replicate number 1	2	3	4	5	Mean % fertilized	SD	Significantly different from control[e]
OB27 (rep OB1)	**100**	98	89	97	95	95	**94.8**	3.49	
OB27 (rep OB1)	**50**	95	97	95	93	98	**95.6**	1.95	
OB27 (rep OB1)	**25**	90	91	96	95	97	**93.8**	3.11	
PT1	**100**	90	97	98	99	98	**96.4**	3.65	
PT1	**50**	93	97	99	98	99	**97.2**	2.49	
PT1	**25**	97	96	100	97	90	**96.0**	3.67	
PT2	**100**	95	92	98	94	96	**95.0**	2.24	
PT2	**50**	94	99	97	98	98	**97.2**	1.92	
PT2	**25**	97	98	96	95	95	**96.2**	1.30	
PT3	**100**	97	98	95	96	98	**96.8**	1.30	
PT3	**50**	97	95	98	94	98	**96.4**	1.82	
PT3	**25**	93	97	98	99	97	**96.8**	2.28	
PT4	**100**	98	98	93	97	95	**96.2**	2.17	
PT4	**50**	95	99	95	98	95	**96.4**	1.95	
PT4	**25**	95	96	92	98	98	**95.8**	2.49	
PT5	**100**	98	97	93	94	91	**94.6**	2.88	
PT5	**50**	95	95	93	95	89	**93.4**	2.61	
PT5	**25**	94	92	92	95	95	**93.6**	1.52	
PT6	**100**	91	95	92	97	98	**94.6**	3.05	
PT6	**50**	98	97	97	94	96	**96.4**	1.52	
PT6	**25**	94	92	94	97	95	**94.4**	1.82	
PT7	**100**	98	97	96	94	93	**95.6**	2.07	
PT7	**50**	90	96	94	94	98	**94.4**	2.97	
PT7	**25**	94	97	98	97	92	**95.6**	2.51	
PT8	**100**	97	99	97	96	96	**97.0**	1.22	
PT8	**50**	95	93	93	95	93	**93.8**	1.10	
PT8	**25**	95	92	92	99	96	**94.8**	2.95	
PT9	**100**	94	95	98	95	97	**95.8**	1.64	
PT9	**50**	97	95	97	97	98	**96.8**	1.10	
PT9	**25**	98	94	97	98	98	**97.0**	1.73	

Appendix D1. Continued.

Station	Dilution	% Fertilized							Significantly different
		Replicate number					Mean		
	(%)	1	2	3	4	5	% fertilized	SD	from control[e]
PT10	**100**	98	89	93	87	95	**92.4**	4.45	
PT10	**50**	96	92	96	94	97	**95.0**	2.00	
PT10	**25**	95	95	91	96	98	**95.0**	2.55	
PT11	**100**	93	95	91	93	96	**93.6**	1.95	
PT11	**50**	94	96	99	98	95	**96.4**	2.07	
PT11	**25**	97	92	90	97	93	**93.8**	3.11	
PT12	**100**	90	95	92	93	94	**92.8**	1.92	
PT12	**50**	96	95	94	100	96	**96.2**	2.28	
PT12	**25**	97	93	91	93	97	**94.2**	2.68	
PT13	**100**	74	84	80	82	86	**81.2**	4.60	
PT13	**50**	95	89	91	89	93	**91.4**	2.61	
PT13	**25**	92	96	96	94	95	**94.6**	1.67	
PT14	**100**	95	93	94	96	97	**95.0**	1.58	
PT14	**50**	93	96	94	98	99	**96.0**	2.55	
PT14	**25**	92	98	94	97	97	**95.6**	2.51	
PT15	**100**	51	53	55	45	54	**51.6**	3.97	**
PT15	**50**	95	92	90	89	90	**91.2**	2.39	
PT15	**25**	89	91	95	94	97	**93.2**	3.19	
PT16	**100**	95	93	94	99	96	**95.4**	2.30	
PT16	**50**	93	94	98	96	95	**95.2**	1.92	
PT16	**25**	95	93	95	92	97	**94.4**	1.95	
PT17	**100**	98	93	94	96	97	**95.6**	2.07	
PT17	**50**	94	95	96	97	94	**95.2**	1.30	
PT17	**25**	96	92	89	95	95	**93.4**	2.88	
PT18	**100**	74[d]	90	96	98	96	**95.0**	3.46	
PT18	**50**	96	94	96	97	98	**96.2**	1.48	
PT18	**25**	97	97	98	96	95	**96.6**	1.14	
PT19	**100**	98	95	97	97	98	**97.0**	1.22	
PT19	**50**	98	98	92	95	95	**95.6**	2.51	
PT19	**25**	97	97	99	94	95	**96.4**	1.95	

Appendix D1. Continued.

Station	Dilution (%)	% Fertilized: Replicate number 1	2	3	4	5	Mean % fertilized	SD	Significantly different from control[e]
PT20	**100**	97	92	89	92	94	**92.8**	2.95	
PT20	**50**	94	99	98	97	98	**97.2**	1.92	
PT20	**25**	94	92	96	97	94	**94.6**	1.95	
PT21	**100**	94	96	95	94	91	**94.0**	1.87	
PT21	**50**	99	97	98	92	96	**96.4**	2.70	
PT21	**25**	95	95	99	98	94	**96.2**	2.17	
PT22	**100**	95	100	97	94	99	**97.0**	2.55	
PT22	**50**	94	93	93	95	93	**93.6**	0.89	
PT22	**25**	96	92	97	97	97	**95.8**	2.17	
PT23	**100**	73	84	69	68	79	**74.6**	6.80	**
PT23	**50**	93	92	96	96	93	**94.0**	1.87	
PT23	**25**	96	97	88	97	94	**94.4**	3.78	
PT24	**100**	95	89	97	95	98	**94.8**	3.49	
PT24	**50**	96	94	97	95	99	**96.2**	1.92	
PT24	**25**	96	95	97	98	95	**96.2**	1.30	
PT25	**100**	92	95	92	96	95	**94.0**	1.87	
PT25	**50**	96	93	93	95	99	**95.2**	2.49	
PT25	**25**	96	99	96	95	91	**95.4**	2.88	
PT26 (rep PT13)	**100**	96	96	96	94	96	**95.6**	0.89	
PT26 (rep PT13)	**50**	98	95	95	97	97	**96.4**	1.34	
PT26 (rep PT13)	**25**	94	95	100	97	97	**96.6**	2.30	
SQ1	**100**	92	84	81	83	81	**84.2**	4.55	
SQ1	**50**	95	94	99	96	97	**96.2**	1.92	
SQ1	**25**	97	96	98	96	97	**96.8**	0.84	
SQ2	**100**	46	45	43	52	64[d]	**46.5**	3.87	**
SQ2	**50**	94	96	94	96	96	**95.2**	1.10	
SQ2	**25**	95	98	96	91	97	**95.4**	2.70	

Appendix D1. Continued.

Station	Dilution (%)	% Fertilized							Significantly different from control[e]
		Replicate number					Mean	SD	
		1	2	3	4	5	% fertilized		
MFS[b]	**100**	90	90	96	94	96	**92.3**	3.30	
MFS	**100**	87	93	88	94	95			
Brine control[c]	**100**	43	36	34	14	14	**28.2**	13.39	

[a] Reference pore water from sediment from Redfish Bay, TX; [b] Millipore filtered seawater; [c] Deionized water adjusted to test salinity by brine addition; [d] data detected as outlier and not included in statistical analysis; [e] * indicates significant difference at alpha ≤ 0.05, and ** indicates significant difference at alpha ≤ 0.01.

Appendix D2. Toxicity data for sea urchin, *Arbacia punctulata,* embryological develoment test with 55 porewater samples from stations at Jackson Park and Port Hadlock sites, and Sequim Bay, Puget Sound.

Station	Dilution (%)	% Normal Development							Significantly different from control[c]
		Replicate number					Mean		
		1	2	3	4	5	% fertilized	SD	
REF[a]	**100**	61	54	59	51	67	57.6	4.77	
REF	**100**	56	53	57	62	56			
REF	**50**	56	60	52	52	48	55.3	4.03	
REF	**50**	59	61	54	56	55			
REF	**25**	70	57	52	57	51	56.9	6.90	
REF	**25**	56	64	45	58	59			
OB1	**100**	3	3	7	1	0	2.8	2.68	**
OB1	**50**	49	61	55	61	55	56.2	5.02	
OB1	**25**	46	52	49	58	63	53.6	6.88	
OB2	**100**	0	0	0	0	0	0.0	0.00	**
OB2	**50**	47	59	59	47	60	54.4	6.77	
OB2	**25**	50	56	67	45	55	54.6	8.20	
OB3	**100**	0	0	0	0	0	0.0	0.00	**
OB3	**50**	54	53	51	41	50	49.8	5.17	
OB3	**25**	71	61	42	57	44	55.0	12.10	
OB4	**100**	0	0	0	0	0	0.0	0.00	**
OB4	**50**	0	0	0	0	0	0.0	0.00	**
OB4	**25**	48	47	63	45	52	51.0	7.18	
OB5	**100**	0	0	0	0	0	0.0	0.00	**
OB5	**50**	24	12	14	34	21	21.0	8.77	**
OB5	**25**	39	50	49	47	42	45.4	4.72	
OB6	**100**	0	0	0	0	0	0.0	0.00	**
OB6	**50**	41	43	48	38	39	41.8	3.96	**
OB6	**25**	38	43	44	56	45	45.2	6.61	*
OB7	**100**	0	0	0	0	0	0.0	0.00	**
OB7	**50**	48	54	51	51	54	51.6	2.51	
OB7	**25**	58	63	60	55	57	58.6	3.05	

Appendix D2. Continued.

Station	Dilution (%)	% Normal Development							Significantly different from control[e]
		Replicate number					Mean		
		1	2	3	4	5	% fertilized	SD	
OB8	**100**	0	0	0	0	0	0.0	0.00	**
OB8	**50**	3	0	1	1	1	1.2	1.10	**
OB8	**25**	40	55	69	60	54	55.6	10.55	
OB9	**100**	0	0	1	0	3	0.8	1.30	**
OB9	**50**	56	49	28[d]	49	50	51.0	3.37	
OB9	**25**	40	48	51	35	53	45.4	7.64	
OB10	**100**	0	0	0	0	0	0.0	0.00	**
OB10	**50**	19	18	15	12	1[d]	16.0	3.16	**
OB10	**25**	51	62	58	57	54	56.4	4.16	
OB11	**100**	0	0	0	0	0	0.0	0.00	**
OB11	**50**	0	0	0	0	0	0.0	0.00	**
OB11	**25**	48	55	40	56	48	49.4	6.47	
OB12	**100**	0	0	0	0	0	0.0	0.00	**
OB12	**50**	0	0	0	0	0	0.0	0.00	**
OB12	**25**	49	47	37	50	46	45.8	5.17	
OB13	**100**	0	0	0	0	0	0.0	0.00	**
OB13	**50**	37	40	45	38	42	40.4	3.21	**
OB13	**25**	48	38	52	56	61	51.0	8.72	
OB14	**100**	0	0	0	0	0	0.0	0.00	**
OB14	**50**	0	0	0	0	0	0.0	0.00	**
OB14	**25**	72	51	42	52	53	54.0	10.98	
OB15	**100**	0	0	0	0	0	0.0	0.00	**
OB15	**50**	0	0	0	0	0	0.0	0.00	**
OB15	**25**	45	43	33	48	44	42.6	5.68	**
OB16	**100**	0	0	0	0	0	0.0	0.00	**
OB16	**50**	70[d]	50	45	54	43	48.0	4.97	
OB16	**25**	52	50	55	42	45	48.8	5.26	

Appendix D2. Continued.

Station	Dilution (%)	% Normal Development: Replicate number 1	2	3	4	5	Mean % fertilized	SD	Significantly different from control[e]
OB17	**100**	1	2	13	5	7	5.6	4.77	**
OB17	**50**	55	37	49	53	63	51.4	9.53	
OB17	**25**	56	76	62	55	49	59.6	10.26	
OB18	**100**	0	0	0	0	0	0.0	0.00	**
OB18	**50**	1	5	0	0	1	1.4	2.07	**
OB18	**25**	47	42	56	58	54	51.4	6.69	
OB19	**100**	0	0	0	0	0	0.0	0.00	**
OB19	**50**	35	48	30	42	47	40.4	7.77	**
OB19	**25**	52	35	53	54	67	52.2	11.39	
OB20	**100**	0	0	0	0	0	0.0	0.00	**
OB20	**50**	41	50	48	48	53	48.0	4.42	
OB20	**25**	51	56	63	53	32	51.0	11.55	
OB21	**100**	57	43	41	43	43	45.4	6.54	**
OB21	**50**	57	64	46	55	53	55.0	6.52	
OB21	**25**	56	59	55	48	56	54.8	4.09	
OB22	**100**	0	0	0	0	0	0.0	0.00	**
OB22	**50**	0	0	0	0	0	0.0	0.00	**
OB22	**25**	43	30	43	36	29	36.2	6.76	**
OB23	**100**	0	0	0	0	0	0.0	0.00	**
OB23	**50**	4	44	7	38	38	26.2	19.08	**
OB23	**25**	48	43	41	47	51	46.0	4.00	
OB24	**100**	0	0	0	0	0	0.0	0.00	**
OB24	**50**	46	38	58	52	55	49.8	7.95	
OB24	**25**	63	51	52	51	49	53.2	5.59	
OB25	**100**	17	15	19	22	23	19.2	3.35	**
OB25	**50**	58	67	50	61	55	58.2	6.38	
OB25	**25**	52	50	60	50	60	54.4	5.18	

Appendix D2. Continued.

Station	Dilution (%)	% Normal Development: Replicate number 1	2	3	4	5	Mean % fertilized	SD	Significantly different from control[e]
OB26 (rep OB8)	**100**	0	0	0	0	0	0.0	0.00	**
OB26 (rep OB8)	**50**	8	4	6	17	1	7.2	6.06	**
OB26 (rep OB8)	**25**	44	53	53	53	58	52.2	5.07	
OB27 (rep OB1)	**100**	0	0	0	0	0	0.0	0.00	**
OB27 (rep OB1)	**50**	51	45	43	48	46	46.6	3.05	
OB27 (rep OB1)	**25**	36	49	48	51	53	47.4	6.66	
PT1	**100**	0	58	50	55	63	56.5	5.45	
PT1	**50**	59	57	56	62	57	58.2	2.39	
PT1	**25**	51	46	51	57	58	52.6	4.93	
PT2	**100**	52	54	50	49	51	51.2	1.92	
PT2	**50**	43	47	57	60	48	51.0	7.18	
PT2	**25**	40	58	48	46	47	47.8	6.50	
PT3	**100**	25	32	1	18	29	26.0	6.06	**
PT3	**50**	44	47	42	54	49	47.2	4.66	
PT3	**25**	61	61	54	53	40	53.8	8.58	
PT4	**100**	46	57	43	48	58	50.4	6.73	
PT4	**50**	50	55	50	54	50	51.8	2.49	
PT4	**25**	57	52	51	61	51	54.4	4.45	
PT5	**100**	36	51	48	41	53	45.8	7.12	*
PT5	**50**	51	47	42	61	43	48.8	7.69	
PT5	**25**	41	45	46	46	54	46.4	4.72	
PT6	**100**	0	0	0	0	0	0.0	0.00	**
PT6	**50**	50	53	56	54	33[d]	53.2	2.50	
PT6	**25**	0	64	56	53	54	56.8	5.00	
PT7	**100**	27	45	33	33	34	34.4	6.54	**
PT7	**50**	39	42	29	49	47	41.2	7.89	**
PT7	**25**	41	31	51	38	51	42.4	8.65	**

Appendix D2. Continued.

Station	Dilution (%)	% Normal Development							Significantly different from control[e]
		Replicate number					Mean	SD	
		1	2	3	4	5	% fertilized		
PT8	**100**	0	0	0	0	0	0.0	0.00	**
PT8	**50**	50	33	48	46	43	44.0	6.67	
PT8	**25**	33	37	55	41	46	42.4	8.53	**
PT9	**100**	43	42	48	43	52	45.6	4.28	**
PT9	**50**	42	35	47	66[d]	53	44.2	7.63	
PT9	**25**	48	56	54	57	62	55.4	5.08	
PT10	**100**	0	0	15[d]	0	0	0.0	0.00	**
PT10	**50**	58	51	55	51	39	50.8	7.22	
PT10	**25**	52	58	63	50	56	55.8	5.12	
PT11	**100**	12[d]	34	26	27	4[d]	29.0	4.36	**
PT11	**50**	40	27	45	42	54	41.6	9.76	**
PT11	**25**	43	47	38	47	36	42.2	5.07	**
PT12	**100**	38	37	49	47	38	41.8	5.72	**
PT12	**50**	38	42	46	35	47	41.6	5.13	**
PT12	**25**	58	35	53	51	58	51.0	9.46	
PT13	**100**	38	38	31	6[d]	45	38.0	5.72	**
PT13	**50**	54	36	46	54	47	47.4	7.40	
PT13	**25**	45	58	53	36	52	48.8	8.53	
PT14	**100**	50	29	24	47	36	37.2	11.21	**
PT14	**50**	44	51	51	54	59	51.8	5.45	
PT14	**25**	55	51	53	47	47	50.6	3.58	
PT15	**100**	16	18	15	26	16	18.2	4.49	**
PT15	**50**	57	51	45	66	54	54.6	7.77	
PT15	**25**	39	50	55	54	54	50.4	6.66	
PT16	**100**	9	3	7	21	24	12.8	9.18	**
PT16	**50**	41	37	42	42	46	41.6	3.21	**
PT16	**25**	50	44	41	52	45	46.4	4.51	

Appendix D2. Continued.

Station	Dilution (%)	% Normal Development							Significantly different from control[e]
		Replicate number					Mean		
		1	2	3	4	5	% fertilized	SD	
PT17	**100**	13	9	6	7	10	9.0	2.74	**
PT17	**50**	50	49	39	42	53	46.6	5.86	
PT17	**25**	54	54	66	47	54	55.0	6.86	
PT18	**100**	0	0	0	0	2	0.4	0.89	**
PT18	**50**	48	42	51	52	44	47.4	4.34	
PT18	**25**	60	57	59	62	52	58.0	3.81	
PT19	**100**	0	0	0	0	0	0.0	0.00	**
PT19	**50**	43	52	43	36	40	42.8	5.89	*
PT19	**25**	37	47	40	45	39	41.6	4.22	**
PT20	**100**	21[d]	0	9[d]	0	0	0.0	0.00	**
PT20	**50**	42	57	41	47	34	44.2	8.53	
PT20	**25**	49	42	55	42	40	45.6	6.27	
PT21	**100**	0	0	0	0	0	0.0	0.00	**
PT21	**50**	43	32	46	49	44	42.8	6.46	*
PT21	**25**	55	46	51	63	44	51.8	7.60	
PT22	**100**	1	0	0	0	0	0.2	0.45	**
PT22	**50**	32	43	46	55	55	46.2	9.58	
PT22	**25**	60	54	47	55	55	54.2	4.66	
PT23	**100**	2	6	1	1	6	3.2	2.59	**
PT23	**50**	50	47	49	54	45	49.0	3.39	
PT23	**25**	56	57	26[d]	1[d]	58	57.0	1.00	
PT24	**100**	0	0	0	0	10[d]	0.0	0.00	**
PT24	**50**	39	49	45	56	60	49.8	8.41	
PT24	**25**	44	48	45	48	38	44.6	4.10	*
PT25	**100**	0	0	0	0	0	0.0	0.00	**
PT25	**50**	74[d]	40	54	54	60	52.0	8.48	
PT25	**25**	52	49	33	55	41	46.0	8.94	

Appendix D2. Continued.

Station	Dilution	% Normal Development							Significantly different
		Replicate number					Mean		
	(%)	1	2	3	4	5	% fertilized	SD	from control[e]
PT26 (rep OB13)	**100**	2	2	3	3	1	2.2	0.84	**
PT26 (rep OB13)	**50**	60	50	55	44	50	51.8	6.02	
PT26 (rep OB13)	**25**	53	52	67	49	51	54.4	7.20	
SQ1	**100**	0	0	0	0	0	0.0	0.00	**
SQ1	**50**	0	0	0	0	0	0.0	0.00	**
SQ1	**25**	46	56	50	46	39	47.4	6.23	
SQ2	**100**	0	0	0	0	0	0.0	0.00	**
SQ2	**50**	0	0	0	0	0	0.0	0.00	**
SQ2	**25**	63	55	58	46	46	53.6	7.50	
MFS[b]	**100**	62	62	60	64	61	59.7	4.64	
MFS	**100**	63	62	49	60	54			
Brine control[c]	**100**	35	41	40	35	42	38.6	3.36	

[a] Reference pore water from sediment from Redfish Bay, TX; [b] Millipore filtered seawater; [c] Deionized water adjusted to test salinity by brine addition; [d] data detected as outlier and not included in statistical analysis; [e] * indicates significant difference at alpha $\leq$0.05, and ** indicates significant difference at alpha $\leq$0.01.

Appendix D3. Water quality measurements for 55 porewater samples from stations at Jackson Park and Port Hadlock sites, and Sequim Bay, Puget Sound, used in urchin fertilization and embryological development tests.

Station	salinity	DO (mg/L)	DO %	pH	NH_4 (mg/L)	NH_3 (ug/L)	Sulfide (mg/L)	% OUS[c]
Brine control[a]	30	7.07	96.5	8.40	0.0007	0.1	<.005	76
REF[b]	26	7.73	102.4	7.99	0.242	7.4	<.005	96
OB1	30	6.83	86.9	7.55	0.92	10.4	<.005	100
OB2	20	8.03	99.9	7.69	1.22	19.0	<.005	90
OB3	30	7.65	93.3	7.61	0.819	10.7	<.005	100
OB4	30	7.84	99.1	7.64	1.91	26.6	<.005	100
OB5	30	6.60	84.2	7.60	1.57	20.0	<.005	100
OB6	30	7.80	101.3	7.55	1.25	14.2	<.005	100
OB7	30	7.23	90.0	7.52	1.10	11.7	<.005	100
OB8	30	6.30	81.0	7.59	1.48	18.4	<.005	100
OB9	28	6.98	93.6	7.61	1.03	13.4	<.005	98
OB10	30	6.22	81.8	7.61	1.66	21.6	<.005	10
OB11	20	7.51	97.8	7.71	2.24	36.6	<.005	91
OB12	30	7.18	95.9	7.68	2.68	40.9	<.005	100
OB13	30	7.49	97.4	7.68	1.79	27.3	<.005	100
OB14	30	6.87	88.9	7.72	2.40	40.1	<.005	100
OB15	30	7.61	93.7	7.73	2.69	45.9	<.005	100
OB16	30	7.33	94.7	7.48	1.29	12.5	<.005	100
OB17	30	7.33	95.8	7.54	1.12	12.4	<.005	100
OB18	28	6.62	84.5	7.62	1.73	23.0	<.005	98
OB19	30	7.46	96.8	7.62	1.59	21.2	<.005	100
OB20	30	7.10	94.7	7.57	1.31	15.6	<.005	100
OB21	14	7.94	106.4	7.78	0.782	15.0	<.005	86
OB22	30	8.23	103.2	7.57	3.25	38.6	<.005	100
OB23	30	6.78	85.1	7.64	2.06	28.7	<.005	100
OB24	30	6.76	87.8	7.51	1.15	11.9	<.005	100
OB25	30	7.44	98.1	7.41	1.08	8.9	<.005	100
OB26 (rep OB8)	30	7.61	101.4	7.58	0.626	7.6	<.005	100
OB27 (rep OB1)	31	7.36	95.9	7.55	1.32	15.0	<.005	100

Appendix D3. Continued

Station	salinity	DO (mg/L)	DO %	pH	NH_4 (mg/L)	NH_3 (ug/L)	Sulfide (mg/L)	% OUS[c]
PT1	31	6.96	91.9	7.37	0.006	0.0	<.005	100
PT2	31	6.89	90.6	7.39	0.616	4.9	<.005	100
PT3	31	7.02	91.8	7.51	1.07	11.1	<.005	100
PT4	31	7.28	97.2	7.38	0.549	4.2	<.005	100
PT5	31	7.00	91.9	7.48	0.655	6.3	<.005	100
PT6	31	7.26	93.5	7.40	1.56	12.6	<.005	100
PT7	31	6.51	82.3	7.53	0.971	10.5	<.005	100
PT8	31	6.31	81.0	7.50	1.51	15.3	<.005	100
PT9	31	7.37	95.8	7.57	1.24	14.7	<.005	100
PT10	32	6.98	91.8	7.46	1.25	11.6	<.005	94
PT11	32	7.12	90.8	7.53	1.01	11.0	<.005	94
PT12	32	6.37	80.3	7.61	1.01	13.1	<.005	94
PT13	32	7.56	94.6	7.52	1.14	12.1	<.005	94
PT14	32	7.63	97.6	7.48	1.25	12.1	<.005	94
PT15	32	7.37	95.8	7.41	1.29	10.6	<.005	94
PT16	31	6.64	85.6	7.62	1.43	19.0	<.005	100
PT17	32	7.49	94.4	7.55	1.30	14.8	<.005	94
PT18	31	6.35	80.2	7.46	1.80	16.6	<.005	100
PT19	31	7.36	94.9	7.39	1.78	14.0	<.005	100
PT20	31	8.09	103.7	7.45	1.44	13.0	<.005	100
PT21	31	6.64	83.7	7.48	2.01	19.5	<.005	100
PT22	31	7.17	90.6	7.61	1.56	20.3	<.005	100
PT23	31	6.64	84.8	7.55	1.67	19.0	<.005	100
PT24	31	6.72	87.4	7.59	1.40	17.4	<.005	100
PT25	31	7.34	95.6	7.62	1.65	22.0	<.005	100
PT26 (rep PT13)	32	7.12	92.1	7.48	1.09	10.5	<.005	94
SQ1	32	7.00	90.4	7.55	3.50	39.7	0.011	94
SQ2	32	5.64	75.1	7.61	2.89	37.6	0.011	94

[a] Deionized water adjusted to test salinity by brine addition; [b] Reference pore water from sediment from Redfish Bay, TX; [c] Percent of unadjsuted sample

Appendix D4. Concentrations of a wide range of chemicals measured in selected sediment samples from Jackson Park and Port Hadlock sites. (ND = not detected)

Chemical	Units	OB-6	OB-8	OB-12	OB-15	OB-16	OB-18	OB-22	OB-23	OB-26 (rep OB8)	PT-7	PT-11	PT-12	PT-16	PT-19
Ordnance Compounds															
HMX	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
RDX	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,3,5-Trinitrobenzene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tetryl	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,3-Dinitrobenzene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,4,6-Trinitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Nitrobenzene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-Amino-2,6-dinitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Amino-4,6-dinitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,6-Dinitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2,4-Dinitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Nitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-Nitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3-Nitrotoluene	mg/Kg (ppm)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Picric Acid	mg/Kg (ppm)	0.2	0.6	0.2	0.7	1.0	0.7	0.4	0.7	0.5	ND	0.3	0.2	0.1	0.2
Butyltins															
Tri-n-butyltin	ug/Kg (ppb)	0.4	3	0.9	2	3	4	2	3	3	ND	0.9	0.7	0.8	ND
Di-n-butyltin	ug/Kg (ppb)	0.5	3	1	2	3	4	2	4	3	ND	1	0.7	0.6	ND
n-Butyltin	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PCBs															
Aroclor 1016	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aroclor 1221	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aroclor 1232	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aroclor 1242	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aroclor 1248	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aroclor 1254	ug/Kg (ppb)	ND	6	ND	10	17	30	15	21	17	ND	ND	ND	ND	ND
Aroclor 1260	ug/Kg (ppb)	ND	6	ND	7	11	16	10	13	11	ND	ND	ND	ND	ND

Appendix D4. Continued

Chemical	Units	OB-6	OB-8	OB-12	OB-15	OB-16	OB-18	OB-22	OB-23	OB-26	PT-7	PT-11	PT-12	PT-16	PT-19
Base Neutral/Acid Semivolatile Organic Compounds															
Phenol	ug/Kg (ppb)	28	520	560	730	1400	1100	860	540	970	19	1200	500	280	300
1,4-Dichlorobenzene	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	2	ND	ND	ND	ND	ND
1,2-Dichlorobenzene	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzyl Alcohol	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Methylphenol	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4-Methylphenol	ug/Kg (ppb)	ND	55	ND	ND	110	170	ND	93	170	ND	120	62	ND	65
2,4-Dimethylphenol	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1,2,4-Trichlorobenzene	ug/Kg (ppb)	ND	ND	ND	ND	ND	2	ND	ND	ND	ND	ND	5	ND	ND
Naphthalene	ug/Kg (ppb)	1	4	3	4	4	6	7	4	6	ND	6	ND	5	2
Benzoic Acid	ug/Kg (ppb)	520	710	ND	ND	ND	ND	ND	ND	ND	ND	830	ND	ND	ND
Hexachlorobutadiene	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-Methylnaphthalene	ug/Kg (ppb)	ND	5	3	5	5	7	7	6	7	ND	6	4	4	ND
Acenaphthylene	ug/Kg (ppb)	ND	9	5	9	8	13	20	11	14	ND	3	3	4	ND
Dimethyl Phthalate	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acenaphthene	ug/Kg (ppb)	ND	3	ND	ND	ND	4	8	3	3	ND	3	3	2	ND
Dibenzofuran	ug/Kg (ppb)	ND	4	2	3	3	6	20	4	6	ND	5	4	3	ND
Fluorene	ug/Kg (ppb)	ND	13	6	6	7	19	18	8	8	ND	5	6	5	ND
Diethyl Phthalate	ug/Kg (ppb)	6	4	4	3	4	6	5	6	5	2	3	4	3	3
N-Nitrosodiphenylamine	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Hexachlorobenzene	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pentachlorophenol	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phenanthrene	ug/Kg (ppb)	8	56	21	23	22	95	560	42	69	ND	26	24	21	4
Anthracene	ug/Kg (ppb)	3	40	28	17	10	69	71	22	41	ND	10	12	14	ND
Di-n-butyl Phthalate	ug/Kg (ppb)	6	ND	6	8	ND	17	12	16	15	ND	ND	4	3	6
Fluoranthene	ug/Kg (ppb)	40	200	56	79	63	430	980	190	240	ND	78	100	120	7
Pyrene	ug/Kg (ppb)	36	210	60	100	88	480	850	210	270	ND	65	95	140	6
Butyl Benzyl Phthalate	ug/Kg (ppb)	ND	ND	ND	13	ND	26	ND	25	19	ND	ND	ND	ND	ND
Benz(a)anthracene	ug/Kg (ppb)	13	110	71	58	43	200	200	84	110	ND	23	28	45	3
Chrysene	ug/Kg (ppb)	26	210	120	68	54	800	400	150	280	ND	27	36	58	ND

Appendix D4. Continued

Chemical	Units	OB-6	OB-8	OB-12	OB-15	OB-16	OB-18	OB-22	OB-23	OB-26	PT-7	PT-11	PT-12	PT-16	PT-19
Base Neutral/Acid Semivolatile Organic Compounds															
Bis(2-ethylhexyl) Phthalate	ug/Kg (ppb)	22	70	58	170	63	78	81	71	96	18	53	64	26	37
Di-n-octyl Phthalate	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzo(b)fluoranthene	ug/Kg (ppb)	19	150	100	53	60	310	310	130	170	ND	23	24	50	2
Benzo(k)fluoranthene	ug/Kg (ppb)	15	120	76	46	47	230	240	100	130	ND	18	21	45	ND
Benzo(a)pyrene	ug/Kg (ppb)	17	140	100	68	62	250	260	110	150	ND	17	19	41	2
Indeno(1,2,3-cd)pyrene	ug/Kg (ppb)	18	140	100	78	78	190	190	95	230	5	24	14	23	4
Dibenz(a,h)anthracene	ug/Kg (ppb)	6	42	22	18	19	46	45	22	81	3	3	4	7	ND
Benzo(g,h,i)perylene	ug/Kg (ppb)	13	75	55	46	45	140	140	76	100	3	11	12	18	2
Organochlorines															
alpha-BHC	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.3
beta-BHC	ug/Kg (ppb)	ND	ND	ND	ND	2	ND	ND	ND	ND	ND	ND	ND	1	ND
gamma-BHC (Lindane)	ug/Kg (ppb)	0.2	ND	ND	ND	ND	ND	ND	2	ND	ND	0.2	ND	0.7	ND
delta-BHC	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Heptachlor	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aldrin	ug/Kg (ppb)	0.2	0.2	ND	ND	0.3	0.3	0.2	0.3	0.3	ND	ND	ND	ND	ND
Heptachlor Epoxide	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	0.7	ND	ND	ND	ND	ND	ND
gamma-Chlordane	ug/Kg (ppb)	ND	0.4	0.5	ND	ND	ND	ND	ND	0.5	ND	0.4	0.3	0.6	ND
Endosulfan I	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
alpha-Chlordane	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.6	ND	0.3	ND
Dieldrin	ug/Kg (ppb)	ND	0.6	ND	0.5	0.8	1	ND	1	0.7	ND	0.8	ND	ND	0.5
4,4'-DDE	ug/Kg (ppb)	0.4	0.6	ND	ND	0.8	1	0.4	1	0.7	ND	0.3	0.7	0.9	ND
Endrin	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Endosulfan II	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4,4'-DDD	ug/Kg (ppb)	ND	ND	ND	0.2	ND	0.5	ND	ND	ND	ND	ND	ND	0.5	ND
Endrin Aldehyde	ug/Kg (ppb)	0.2	0.7	0.4	0.4	0.5	1	0.5	0.8	2	ND	ND	ND	ND	ND
Endosulfan Sulfate	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4,4'-DDT	ug/Kg (ppb)	0.3	0.7	0.4	1	ND	3	0.9	1	2	ND	2	0.4	0.7	ND

Appendix D4. Continued

Chemical	Units	OB-6	OB-8	OB-12	OB-15	OB-16	OB-18	OB-22	OB-23	OB-26	PT-7	PT-11	PT-12	PT-16	PT-19
Organochlorines															
Endrin Ketone	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.4	0.6	1	ND
Methoxychlor	ug/Kg (ppb)	ND	ND	ND	ND	3	1	ND	ND	ND	ND	ND	ND	ND	ND
Toxaphene	ug/Kg (ppb)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Metals															
Arsenic	mg/Kg (ppm)	ND	12	5	7	9	8	5	10	6	1	4	3	3	2
Cadmium	mg/Kg (ppm)	ND	2.1	0.4	0.4	1.7	1.2	0.8	1	1.2	ND	0.3	0.4	0.3	0.5
Chromium	mg/Kg (ppm)	1	61	19	30	38	34	26	42	38	18	23	18	19	17
Copper	mg/Kg (ppm)	1.1	102	16.6	32.4	50.1	74.9	90.3	53.2	62.2	5.3	14.8	8.4	9.4	6.6
Lead	mg/Kg (ppm)	0.9	79.8	19.4	35.8	45.4	49.8	39.2	62.9	40.7	1.2	9.5	4.7	8.9	2.4
Mercury	mg/Kg (ppm)	0.1	0.3	0.1	0.2	0.4	0.3	0.2	0.3	0.3	ND	0.05	0.02	0.03	0.02
Silver	mg/Kg (ppm)	0.1	0.5	0.2	0.3	0.6	0.7	0.3	0.5	0.6	0.03	0.1	0.05	0.05	0.04
Zinc	mg/Kg (ppm)	3	169	46	73	100	111	92	100	99	19	44	30	34	24
Total Solids	Percent	78.5	48.8	62.4	43.3	38.9	40.6	53.8	37.6	43.5	80.2	58.0	75.7	76.4	76.2

Appendix D5. Grains size distribution in selected sediment samples from Jackson Park and Port Hadlock sites, Puget Sound.

Station	% Gravel	% Sand	% Silt	% Clay	Total
OB-6	0.13	86.79	6.77	5.86	**99.5**
OB-8	0.62	23.79	55	20.7	**100**
OB-12	1.47	72.52	15.3	9.5	**98.8**
OB-15	1.21	44.27	34.8	19.1	**99.4**
OB-16	0.19	10.05	65.7	21.2	**97.1**
OB-18	0	20.56	58.7	17.6	**96.9**
OB-22	21.5	38.15	33.5	12.7	**106.0**
OB-23	0.05	12.86	63.9	23.3	**100.0**
OB-26 (rep OB8)	1.34	23.53	53.8	18.1	**96.7**
PT-7	0.0	102.25	0.04	0.76	**103.0**
PT-11	0.62	64.55	26.8	12.6	**104.0**
PT-12	0.57	84.05	8.27	6.0	**98.9**
PT-16	0.28	88.96	7.35	4.3	**101.0**
PT-19	1	82.54	9.1	5.1	**97.7**

Appendix E

Complete data set for toxicity tests and analyses conducted for TIE procedure with a composite sample from Jackson Park site, Puget Sound

Appendix E1. Toxicity data for sea urchin, *A. punctulata*, fertilization and embryological development tests with fresh and frozen pore water from sample selected for TIE procedure.

Appendix E2. Water quality data for fresh and frozen pore water from sample selected for TIE procedure.

Appendix E3. Concentrations of a wide range of chemicals in the frozen porewater sample used for the TIE procedure, and in a fresh sample of the same pore water.

Appendix E4. Toxicity data for sea urchin, *A. punctulata*, fertilization test following TIE procedures.

Appendix E5. Data for sea urchin, *A. punctulata*, embryological development test following TIE procedures.

Appendix E6. Water quality data for pore water baseline samples prior to TIE procedures, pH and ammonia measured after the application of selected TIE procedures, and pH measurements at beginning and end of embryological development test for samples submitted to pH adjustment.

Appendix E1. Toxicity data for sea urchin, *A. punctulata*, fertilization and embryological development tests with fresh and frozen pore water from sample selected for TIE procedure. Test date: 8-17-98.

Station	Test	% Dilution	Replicate number 1	2	3	4	5	MEAN	SD	Sign. Diff.	EC_{50}
REF[a]	Fert.	**100**	95	95	96	93	94	**94.6**	1.14		
REF	Fert.	**50**	96	93	94	99	94	**95.2**	2.39		
REF	Fert.	**25**	93	92	100	91	95	**94.2**	3.56		
MFS[b]	Fert.	**100**	89	96	92	99	94	**94**	3.81		
Fresh	Fert.	**100**	18	7	15	4	19	**12.6**	6.73	**	
Fresh	Fert.	**50**	29	44	46	50	36	**41**	8.43	**	**29.2**
Fresh	Fert.	**25**	59	47	55	40	45	**49.2**	7.69	**	**(15.7-54.3)**
Frozen	Fert.	**100**	7	7	6	6	9	**7**	1.22	**	
Frozen	Fert.	**50**	40	54	45	54	47	**48**	6.04	**	**44.7**
Frozen	Fert.	**25**	67	67	55	42	36	**53.4**	14.19	**	**(27.9-71.6)**
REF	Embryo	**100**	51	68	39	38	33	**45.8**	14.06		
REF	Embryo	**50**	36	39	48	43	37	**40.6**	4.93		
REF	Embryo	**25**	29	23	25	23	36	**27.2**	5.50		
MFS	Embryo	**100**	45	34	48	31	43	**40.2**	7.33		
Fresh	Embryo	**100**	0	0	0	0	0	**0**	0.00	**	
Fresh	Embryo	**50**	0	0	0	0	0	**0**	0.00	**	**35.4**
Fresh	Embryo	**25**	53	57	51	72	38	**54.2**	12.24		**(NR)**
Frozen	Embryo	**100**	0	0	0	0	0	**0**	0.00	**	
Frozen	Embryo	**50**	0	0	0	0	0	**0**	0.00	**	**35.4**
Frozen	Embryo	**25**	66	49	47	62	67	**58.2**	9.52		**(NR)**

[a] Reference pore water from sediment from Redfish Bay, TX; [b] Millipore filtered seawater

Appendix E2. Water quality data for fresh and frozen pore water from sample selected for TIE procedure.

Sample	Salin.	DO	DO	pH	NH_4	NH_3	Sulf.
	(ppt)	(mg/L)	% sat		(mg/L)	(ug/L)	(mg/L)
REF	36	8.22	102	7.84	0.782	17.1	<0.1
Fresh	30	2.26[a]	27.500	7.66	7.14	104.1	<0.1
Frozen	30	8.4	106	7.69	7.0	109.5	<0.1

[a] This sample was stirred on magnetic stirrer until DO raised to 7.19 mg/L and 99.9% saturation.

Appendix E3. Concentrations of a wide range of chemicals in the frozen pore water sample used for the TIE procedure, and in a fresh sample of the same pore water.

Chemical	Concentration in fresh or frozen porewater (ug/L)	
	Frozen	Fresh
Ordnance Compounds		
HMX	ND	ND
RDX	ND	ND
1,3,5-Trinitrobenzene	ND	ND
Tetryl	ND	ND
1,3-Dinitrobenzene	ND	ND
2,4,6-Trinitrotoluene	ND	ND
Nitrobenzene	ND	ND
4-Amino-2,6-dinitrotoluene	ND	ND
2-Amino-4,6-dinitrotoluene	ND	ND
2,6-Dinitrotoluene	ND	ND
2,4-Dinitrotoluene	ND	ND
2-Nitrotoluene	ND	ND
4-Nitrotoluene	ND	ND
3-Nitrotoluene	ND	ND
Picric Acid	ND	ND
Butyltins		
Tetra-n-butyltin	0.014	ND
Tri-n-butylltin	ND	ND
Di-n-butyltin	0.029	0.024
n-Butyltin	0.075	0.096
Polychlorinated Biphenyls (PCBs)		
Aroclor 1016	ND	ND
Aroclor 1221	ND	ND
Aroclor 1232	ND	ND
Aroclor 1242	ND	ND
Aroclor 1248	ND	ND
Aroclor 1254	ND	ND
Aroclor 1260	ND	ND
Base Neutral/Acid Semivolatile Organic Compounds		
Phenol	1.5	1.5
1,4-Dichlorobenzene	ND	ND
1,2-Dichlorobenzene	ND	ND
Benzyl Alcohol	ND	ND
2-Methylphenol	ND	ND
4-Methylphenol	ND	ND
2,4-Dimethylphenol	ND	ND
1,2,4-Trichlorobenzene	ND	ND
Naphthalene	0.03	0.03
Benzoic Acid	ND	ND
Hexachlorobutadiene	ND	ND
2-Methylnaphthalene	ND	ND

Appendix E3. Continued

Chemical	Concentration in fresh or frozen porewater (ug/L)	
	Frozen	Fresh
Base Neutral/Acid Semivolatile Organic Compounds		
Acenaphthylene	ND	ND
Dimethyl Phthalate	ND	ND
Acenaphthene	ND	ND
Dibenzofuran	ND	ND
Fluorene	ND	ND
Diethyl Phthalate	0.2	0.2
N-Nitrosodiphenylamine	ND	ND
Hexachlorobenzene	ND	ND
Pentachlorophenol	ND	ND
Phenanthrene	0.05	0.05
Anthracene	ND	ND
Di-n-butyl Phthalate	4.3	4.4
Fluoranthene	ND	ND
Pyrene	ND	ND
Butyl Benzyl Phthalate	0.06	0.05
Benz(a)anthracene	ND	ND
Chrysene	ND	ND
Bis(2-ethylhexyl) Phthalate	3	0.2
Di-n-octyl Phthalate	ND	ND
Benzo(b)fluoranthene	ND	ND
Benzo(k)fluoranthene	ND	ND
Benzo(a)pyrene	ND	ND
Indeno(1,2,3-cd)pyrene	0.07	ND
Dibenz(a,h)anthracene	0.08	ND
Benzo(g,h,i)perylene	0.07	ND
Metals		
Arsenic	4.3	8.0
Cadmium	0.04	0.08
Chromium	ND	0.6
Copper	0.2	0.3
Lead	0.06	0.14
Mercury	ND	ND
Silver	ND	ND
Zinc	0.6	1.1

Appendix E4. Toxicity data for sea urchin, *A. punctulata*, fertilization test following TIE procedures. Test date: 9-04-98.

Treatment	Sample	% Dilution	% Fertilized							Significant Difference[d]
			Replicate number							
			1	2	3	4	5	MEAN	SD	
Baseline	OB[a]	100	21	34	12	16	29	22.4	9.07	
Baseline	OB	50	38	48	29	42	45	40.4	7.37	
Baseline	OB	25	72	84	78	73	67	74.8	6.46	
Baseline	OB	12.5	91	91	95	98	95	94	3.00	
Baseline	OB	6.25	95	96	94	95	96	95.2	0.84	
Baseline	REF[b]	100	84	89	89	87	90	87.8	2.39	
Baseline	REF	50	89	92	91	90	96	91.6	2.70	
Baseline	REF	25	97	96	89	95	88	93	4.18	
Baseline	REF	12.5	95	98	92	95	99	95.8	2.77	
Baseline	REF	6.25	95	96	95	89	94	93.8	2.77	
Baseline	MFS[c]	100	81	98	91	92	80	89.7	5.81	
Baseline		100	91	87	95	88	94			
Aeration	OB	100	12	8	33	14	17	16.8	9.63	
Aeration	OB	50	27	13	34	22	42	27.6	11.10	
Aeration	OB	25	57	81	71	84	73	73.2	10.55	
Aeration	MFS	100	74	82	89	87	87	83.8	6.06	
Filtration	OB	100	3	33	38	26	50	30	17.45	
Filtration	OB	50	57	27	19	74	36	42.6	22.57	
Filtration	OB	25	81	82	79	88	75	81	4.74	
Filtration	MFS	100	85	59	93	88	92	83.4	14.01	
C18	OB	100	64	38	72	37	69	56	17.13	**
C18	OB	50	89	81	68	88	43	73.8	19.15	**
C18	OB	25	87	92	83	93	91	89.2	4.15	**
C18	MFS	100	87	82	82	86	84	84.2	2.28	
EDTA	OB	100	48	43	41	46	45	44.6	2.70	*
EDTA	OB	50	39	36	32	54	52	42.6	9.84	
EDTA	OB	25	81	79	72	89	84	81	6.28	
EDTA	MFS	100	94	92	97	93	93	93.8	1.92	
Na thiosulfate	OB	100	15	28	52	40	29	32.8	13.92	
Na thiosulfate	OB	50	27	22	44	28	39	32	9.14	
Na thiosulfate	OB	25	80	65	74	70	80	73.8	6.50	
Na thiosulfate	MFS	100	95	93	91	87	92	91.6	2.97	

Appendix E4. Continued

Treatment	Sample	% Dilution	% Fertilized							Significant Difference[d]
			Replicate number					MEAN	SD	
			1	2	3	4	5			
pH 7.2	OB	100	27	22	25	32	19	25	4.95	
pH 7.2	OB	50	41	26	28	16	19	26	9.72	
pH 7.2	OB	25	40	41	30	52	33	39.2	8.53	**
pH 7.2	REF	100	49	61	64	68	59	60.2	7.12	
pH 7.2	REF	50	81	83	76	91	89	84	6.08	
pH 7.2	REF	25	86	73	81	89	83	82.4	6.07	
pH 7.2	MFS	100	81	85	90	91	82	85.8	4.55	
pH 8.0	OB	100	26	17	13	15	44	23	12.75	
pH 8.0	OB	50	29	42	17	28	30	29.2	8.87	
pH 8.0	OB	25	58	78	66	59	75	67.2	9.09	
pH 8.0	REF	100	27	32	58	33	40	38	12.10	
pH 8.0	REF	50	87	82	86	83	85	84.6	2.07	
pH 8.0	REF	25	89	92	90	89	92	90.4	1.52	
pH 8.0	MFS	100	93	93	90	95	93	92.8	1.79	
pH 9.0	OB	100	0	0	0	0	0	0	0.00	**
pH 9.0	OB	50	0	0	0	0	0	0	0.00	**
pH 9.0	OB	25	12	13	10	17	14	13.2	2.59	**
pH 9.0	REF	100	18	9	11	6	15	11.8	4.76	
pH 9.0	REF	50	49	36	67	76	69	59.4	16.44	
pH 9.0	REF	25	70	68	75	82	83	75.6	6.80	
pH 9.0	MFS	100	90	88	87	94	88	89.4	2.79	

[a] Pore water from site selected for TIE, from Ostrich Bay; [b] Reference pore water, from Redfish Bay, TX; [c] Millipore filtered seawater; [d] Significanlty different from Ostrich Bay baseline toxicity, * indicates significant difference at alpha ≤ 0.05 and ** indicates significant difference at alpha ≤ 0.01.

Appendix E5. Data for sea urchin, *A. punctulata,* embryological development test following TIE procedures. Test date: 9-04-98.

Treatment	Sample	% Dilution	% Normal Development							Significant Difference[d]
			Replicate number					MEAN	SD	
			1	2	3	4	5			
Baseline	OB[a]	100	0	0	0	0	0	0.0	0.00	
Baseline	OB	50	0	0	0	0	0	0.0	0.00	
Baseline	OB	25	5	19	4	2	5	7.0	6.82	
Baseline	OB	12.5	90	85	89	83	92	87.8	3.70	
Baseline	OB	6.25	87	88	96	88	91	90.0	3.67	
Baseline	REF[b]	100	93	91	88	90	93	91.0	2.12	
Baseline	REF	50	88	92	88	94	97	91.8	3.90	
Baseline	REF	25	93	86	91	94	83	89.4	4.72	
Baseline	REF	12.5	87	92	88	87	94	89.6	3.21	
Baseline	REF	6.25	89	89	91	91	87	89.4	1.67	
Baseline	MFS[c]	100	90	86	90	88	91	89.4	3.27	
Baseline	MFS	100	88	95	94	87	85			
Filtration	OB	100	0	0	0	0	0	0.0	0.00	
Filtration	OB	50	0	0	0	0	0	0.0	0.00	
Filtration	OB	25	5	0	11	36	13	13.0	13.84	
Filtration	MFS	100	95	88	95	89	92	91.8	3.27	
Aeration	OB	100	0	0	0	0	0	0.0	0.00	
Aeration	OB	50	0	0	0	0	0	0.0	0.00	
Aeration	OB	25	0	0	12	3	4	3.8	4.92	
Aeration	MFS	100	86	89	87	89	85	87.2	1.79	
EDTA	OB	100	0	0	0	0	0	0.0	0.00	
EDTA	OB	50	0	0	0	0	0	0.0	0.00	
EDTA	OB	25	85	94	61	84	79	80.6	12.22	**
EDTA	MFS	100	83	80	85	93	88	85.8	4.97	
C18	OB	100	0	0	0	0	0	0.0	0.00	
C18	OB	50	0	0	0	0	0	0.0	0.00	
C18	OB	25	43	63	53	48	65	54.4	9.48	**
C18	MFS	100	92	91	93	88	90	90.8	1.92	
Na thiosulfate	OB	100	0	0	0	0	0	0.0	0.00	
Na thiosulfate	OB	50	0	0	0	0	0	0.0	0.00	
Na thiosulfate	OB	25	36	50	25	59	60	46.0	15.18	**
Na thiosulfate	MFS	100	86	81	91	87	86	86.2	3.56	
pH 7.2	OB	100	0	0	0	0	0	0.0	0.00	
pH 7.2	OB	50	0	4	0	0	5	1.8	2.49	**
pH 7.2	OB	25	91	85	94	90	90	90.0	3.24	**
pH 7.2	REF	100	94	93	95	94	87	92.6	3.21	
pH 7.2	REF	50	88	92	92	87	87	89.2	2.59	
pH 7.2	REF	25	98	86	94	94	87	91.8	5.12	
pH 7.2	MFS	100	91	78	87	92	88	87.2	5.54	

Appendix E5. Continued

Treatment	Sample	% Dilution	% Normal Development							Significant Difference[d]
			Replicate number					MEAN	SD	
			1	2	3	4	5			
pH 8.0	OB	**100**	0	0	0	0	0	**0.0**	0.00	
pH 8.0	OB	**50**	0	0	0	0	0	**0.0**	0.00	
pH 8.0	OB	**25**	43	60	20	26	25	**34.8**	16.54	**
pH 8.0	REF	**100**	96	91	89	87	91	**90.8**	3.35	
pH 8.0	REF	**50**	88	95	93	86	87	**89.8**	3.96	
pH 8.0	REF	**25**	94	82	93	88	88	**89.0**	4.80	
pH 8.0	MFS	**100**	90	93	87	93	85	**89.6**	3.58	
pH 9.0	OB	**100**	0	0	0	0	0	**0.0**	0.00	
pH 9.0	OB	**50**	0	0	0	0	0	**0.0**	0.00	
pH 9.0	OB	**25**	0	0	0	0	0	**0.0**	0.00	
pH 9.0	REF	**100**	0	0	0	0	0	**0.0**	0.00	
pH 9.0	REF	**50**	38	27	22	34	27	**29.6**	6.35	
pH 9.0	REF	**25**	86	92	83	89	91	**88.2**	3.70	
pH 9.0	MFS	**100**	93	94	95	91	85	**91.6**	3.97	

[a] Pore water from site selected for TIE, from Ostrich Bay; [b] Reference pore water, from Redfish Bay, TX;

[c] Millipore filtered seawater; [d] Significanlty different from Ostrich Bay baseline toxicity, ** indicates significant difference at alpha $\leq$ 0.01.

Appendix E6. Water quality data for pore water baseline samples prior to TIE procedures, pH and ammonia measured after the application of selected TIE procedures.

Treatment	Sample	pH	NH_4 (mg/L)	NH_3 (ug/L)	Salinity (ppt)	DO (mg/L)	DO % sat
Baseline	**OB**	7.86	8.140	186.4	30	8.02	102
Baseline	**REF**	8.06	0.626	22.4	26	8.65	110
Baseline	**MFS**	7.99	0.000	0.0	30	7.20	99
Filtration	**OB**	7.74	5.040	88.1			
Filtration	**MFS**	7.94	0.000	0.0			
Aeration	**OB**	8.12	4.380	179.2			
Aeration	**MFS**	7.82	0.000	0.0			
C18	**OB**	7.81	6.720	137.5			
C18	**MFS**	7.41	0.000	0.0			

pH measurements at beginning and end of embryological development test forsamples submited to pH adjustment.

pH Treatment	Sample	Initial pH	Final pH*
pH 7.2	**OB**	7.23	7.72
pH 7.2	**REF**	7.25	7.49
pH 7.2	**MFS**	7.14	7.46
pH 8.0	**OB**	7.98	8.08
pH 8.0	**REF**	8.06	7.70
pH 8.0	**MFS**	7.99	7.64
pH 9.0	**OB**	8.96	8.78
pH 9.0	**REF**	8.99	8.60
pH 9.0	**MFS**	8.98	8.56

ATTACHMENTS 1-7

Attachment 1. (SOP F10.14) Preparation of Filtered (0.45 µm) Seawater.

Attachment 2. (SOP F10.6) Sea Urchin Fertilization Toxicity Test.

Attachment 3. (SOP F10.7) Sea Urchin Embryological Development Toxicity Test.

Attachment 4. (SOP F10.10) *Dinophilus gyrociliatus* Toxicity Test.

Attachment 5. (SOP F10.23) Algal Zoospore Germination and Germling Growth Toxicity Test Protocol.

Attachment 6. (SOP F10.9) Extraction and Storage of Porewater Samples.

Attachment 7. (SOP F10.12) Water Quality Adjustment of Samples.

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and is intended for reference only.

Date Prepared : April 10, 1990

Date Revised: March 10, 1995

SEA URCHIN FERTILIZATION TOXICITY TEST

1.0 OBJECTIVE

The purpose of the fertilization toxicity test with the sea urchin, *Arbacia punctulata*, is to determine if a sea water, pore water, sea surface microlayer, or other sample reduces fertilization of exposed gametes relative to that of gametes exposed to a reference sample. The test may also be used to determine the concentration of a test substance which reduces fertilization. Test results are reported as treatment (or concentration) which produces statistically significant reduced fertilization or as concentration of test substance which reduces fertilization by 50 percent (EC_{50}). This test can be performed concurrently with Sea Urchin Embryological Development Toxicity Test (SOP 10.7) and/or Sea Urchin Genotoxicity/Teratogenicity Test (SOP 10.8), using the same pretest and sperm and egg collection.

2.0 TEST PREPARATION

2.1 Test Animals

Gametes from the sea urchin, *Arbacia punctulata* are used in the sea urchin fertilization toxicity test. Animals can be collected in the field or obtained from a commercial supplier. *A. punctulata* can be differentiated from other species of urchins which are found in Texas by the five plates surrounding the anal opening, and by round sharp spines on the dorsal surface of the test and flattened spines surrounding the Aristotle's lantern. Urchins can be maintained easily in aquaria or other tanks with running seawater or an aquarium filter. Urchins will eat a wide variety of marine vegetation. A good diet may be provided by placing rocks from jetties (which have been colonized by diatoms and macroalgae) into the tank with the urchins or romaine lettuce may be provided as a substitute. Temperature manipulations of the cultures will prolong the useful life of the urchins. Cultures are maintained at $16 \pm 1°C$ when gametes are not required. Temperature is gradually increased to $19 \pm 1°C$ at least one week prior to gamete collection and subsequently decreased if no further tests are planned. Photoperiod is maintained at 16 hours of light per day. Water quality parameters should be monitored weekly and salinity maintained at 30 ± 3 ‰. Males and females should be kept in separate tanks.

2.2 Dilution Water

HPLC reagent grade purified water or concentrated seawater brine is used to adjust samples to 30 ‰ as described in Water Quality Adjustment of Samples (SOP 10.12). Concentrated seawater brine (90-110 ‰) is made in large batches by heating seawater to 40°C or less in large tanks with aeration for 3-4 weeks. Brine quality will remain constant over long periods with no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen are also measured. Salinity adjustment and water quality data are recorded on prepared data forms.

Filtered (0.45 µm) seawater adjusted to 30 ‰ is used to wash eggs and is also used for sperm and egg dilutions. The acronym MFS (for Millipore® filtered seawater) is used for this filtered and salinity adjusted seawater.

2.3 Test System: Equipment

When testing samples for potential toxicity, five replicates per treatment are recommended. One replicate is a 5 mL volume of sample in a disposable glass scintillation vial. When conducting a dilution series test, fifty percent serial dilutions may be made in the test vials, using MFS as the diluent.

2.3.1 Equipment

A list of equipment necessary for conducting this test is given in Attachment 1 (Equipment List for Fertilization Toxicity Test).

2.3.2 Solutions

<u>10% Buffered Formalin</u>:

1,620 mL sea water
620 mL formaldehyde
6.48 g NaH_2P0_4 or KH_2PO_4 (mono)
10.5 g Na_2HPO_4 or K_2HPO_4 (dibasic)

1 mL needed for each replicate. Fill the dispenser.

2.4 Collection and Preparation of Gametes

Quality gametes must first be collected, and then diluted to the appropriate concentration for addition to the test vials.

2.4.1 Selection of Urchins to be Used in Toxicity Test.

1. Take two or three females and place in shallow bowl, barely covering tests with seawater.

2. Stimulate release of eggs from gonopores of a female by touching test with electrodes from a 12V transformer.

3. Collect a few eggs from between spines using a 10 mL disposable syringe with a large gauge blunt-tipped needle attached. Discard the first small quantity of eggs expelled from each gonopore and continue collecting. Place a 2 to 5 drops of eggs onto a scintillation vial containing 10ml of filtered seawater. Rinse syringe and repeat for each female.

4. Select females which have round, well developed eggs, and which do not release clumps of eggs or undeveloped ovarian tissue.

5. Place 2-4 males in shallow bowl(s) with a small amount of seawater, leaving the upper $^1/_2$ to $^1/_3$ of the animals uncovered.

6. Stimulate release of sperm from gonopores by touching test with electrodes from 12V transformer (about 30 seconds each time). If sperm is watery, reject the animal and choose another. Sperm should be the consistency of condensed milk. Collect sperm using a pastuere pipette with a rubber bulb attached.

Generally, a gamete check is performed in order to ensure that both the male and the female urchins used in the test have gametes with a high degree of viability. If the gamete check is performed, two to five females (depending on confidence in the proportion of urchins in the holding facility in good reproductive status) and at least two males should be selected using the above procedures. The check is performed by adding 5 to 7 drops of a concentrated dilution of sperm to the eggs in the scintillation vials (collected as described above) and observing the eggs under the microscope after 10 minutes. The concentrated dilution of sperm is usually made by diluting 20-50µl of sperm in 10 ml of filtered seawater. If the proportion of eggs fertilized is high (95-100%), that female and male may be used in the pretest and test. Sperm from a number of males or females may be combined in the beginning if the gamete check reveals a number of high quality animals or the confidence is high in the quality of the gametes Once a good male and female are selected a pretest can be conducted to determine the correct dilution of sperm to use in the test (Attachment 2).

2.4.2 Obtain Eggs

1. Place selected female in large Carolina dish and add enough water to cover the urchin's test with approximately 1 cm of seawater. Stimulate release of eggs from female with 12V transformer.

2. Collect eggs as above using the 10 mL syringe. Remove needle before dispensing eggs into a disposable shell vial or other clean container capable of holding 25-50 mL. Collect enough eggs for pretest and test. If female stops giving eggs readily or starts giving chunky material, cease stimulation and collection of eggs from that female.

3. Add MFS to fill shell vials, gently mixing eggs. Allow eggs to settle to bottom of vial. Remove water with a pipette. Replace water, again gently mixing the eggs.

4. Repeat washing procedure.

2.4.3 Prepare Appropriate Egg Concentration

1. Put approximately 100 mL of 30 $^{0}/_{00}$ MFS in a 250 mL beaker, and add enough washed eggs to bring the egg density to approximately 10,000 per mL . If more than 400 total replicates (27 treatments) are to be tested, a larger amount of water and a correspondingly larger amount of eggs should be used. Two hundred µL of this egg solution will be used per replicate, and it is easier to maintain proper mixing and uniform egg density if there is an excess of at least 50%.

2. Check egg density and adjust to within approximately 9000 to 11,000 eggs per mL, as follows. Gently swirl egg solution until evenly mixed. Using a pipette, add 1 mL of the solution to a vial containing nine mL seawater. Mix and transfer 1 mL of this diluted solution to a second vial containing 4 mL of seawater. Again, mix and transfer 1 mL of this diluted solution to a counting slide such as a Sedgewick-Rafter slide.

3. Using a microscope (either a compound microscope with a 10x objective or a dissecting scope may be used here), count the number of eggs on the slide. If the number is not between 180 and 220, then adjust by adding eggs or water. If egg count is > 220 use the following formula to calculate the amount of water to add:

   ("egg count" - 200/200) x Current Volume of Eggs = Volume seawater to add to stock (mLs)

   If egg count < 200 add a small amount of eggs. Since it is less arbitrary and more likely to arrive at an acceptable count when using the water addition formula, it is better to originally overestimate the amount of eggs to add to the 100 mL of water.

4. Repeat steps 2 and 3 until an acceptable egg count (between 180 and 220) is obtained.

2.4.4 Obtain Sperm

Place selected male urchin in a large Carolina dish containing 1-2 cm of water. About half of test should be above water level. Stimulate male with 12V transformer, and collect about 0.5 mL of unwetted sperm from between spines using a pasteur pipette. Place sperm into a plastic microcentrifuge tube. Keep on ice until used. Be careful not to add any water or sperm which has contacted water to the vials. High quality sperm collected dry and kept on ice will last at least eight hours without measurable decline in viability.

2.4.5 Prepare Appropriate Sperm Dilution

It is desirable for control fertilization to be within 60-90%. Although controls outside these bounds do not automatically disqualify a test, particularly if a valuable dose response is generated, the sensitivity of the test is reduced by fertilization rates greater than 90% and good dose responses may be difficult to obtain with less than 60% fertilization in controls. Density of sperm in the sperm solution should be determined with this goal in mind. Condition of the animals and length of acclimation to the aquarium may effect the chosen sperm density. The pretest (Attachment 2) may be used to calculate an appropriate sperm dilution. Generally, a dilution of between 1:10,000 and 1:2500 will result in desirable fertilization rates, if the animals are in good condition.

For example, if a sperm dilution of 1:5000 is required (as determined from the pretest), add 20 µL sperm to 10 mL MFS. Mix thoroughly, then add 1 mL of this solution to 9 mL MFS. Sperm should not be wetted until just before starting the test. Sperm wetted more than 30 minutes before the test has begun, including sperm dilutions used in any pretest, should be discarded and a new dilution made from sperm kept on ice.

3.0 TEST PROCEDURES

1. Add 50 µL appropriately diluted sperm to each vial. Record time of sperm addition. Sperm should be used within 30 minutes of wetting.

2. Incubate all test vials at 20 ± 2°C for 30 minutes. At this point it is useful to set a timer for five to ten minutes prior to the end of the incubation period. This will notify the worker early enough to be ready to start the next step exactly on time.

3. While gently swirling the egg solution to maintain even mixing of eggs, use a 200 µL pipetter to add 200 µL diluted egg suspension to each vial. Pipette tips are cut back using

a clean razor blade to prevent crushing the eggs during pipetting. Record time of egg addition.

4. Incubate for 30 minutes at 20 ± 2°C. The timer may be used again at this point.

5. Using the dispenser, add 1 mL of 10% buffered formalin to each sample.

6. Vials may now be capped and stored overnight or for several days until evaluated. Fertilization membranes are easiest to see while eggs are fairly fresh, so evaluation within two to three days may decrease the time required for evaluation.

7. If it is not possible to make the evaluations within several days or the membranes are difficult to discern, an optional technique may be employed. Make up a 200 ‰ NaCl solution (pickling salt) and add 2 to 4 drops of the solution to a 1 mL egg sample on a microscope slide. This solution causes the egg, but not the membrane, to shrink briefly thereby making the membrane easier to see. The effect only lasts for a short time (~5 min.) so the observations must be made immediately after the NaCl solution is added. If this optional technique is employed, it must be used on all samples in that test series.

4.0 DATA COLLECTION AND TABULATION

1. Transfer approximately 1 mL eggs and water from <u>bottom</u> of test vials to counting slide. Observe eggs using compound microscope under 100X magnification. Dark field viewing is useful here in identifying fertilization membranes.

2. Count 100 eggs/sample using hand counter with multiple keys (such as a blood cell counter), using one key to indicate fertilized eggs and another to indicate unfertilized eggs. Fertilization is defined by the presence of fertilization membrane surrounding egg.

3. Calculate fertilization percentage for each replicate test:

$$\frac{\text{Total No. Eggs - No. Eggs Unfertilized}}{\text{Total No. Eggs}} \times 100 = \text{Percent Eggs Fertilized}$$

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments 3-7). Normally, percent fertilization in each treatment is compared to an appropriate reference treatment (seawater, pore water or sea surface microlayer from an uncontaminated environment). Statistical comparisons are made using analysis of variance (ANOVA) and Dunnett's *t*-test (Sokal and Rohlf 1981) on the arc sine square root transformed data. For multiple comparisons among treatments, Ryan's Q test (Day and Quinn 1989) with the arc sine square root transformed data is recommended. The trimmed Spearman-Karber method with Abbott's correction is recommended to calculate EC_{50} values for dilution series tests (Hamilton et al. 1977)

6.0 QUALITY CONTROL

Quality control tests may be run using both positive and negative controls with multiple replicates (as many as desired). Typically, a reference toxicant dilution series (sodium dodecyl sulfate) is tested with each test to evaluate the effectiveness of the sperm dilution chosen. Negative controls may include a reference porewater, filtered seawater, and/or a reconstituted brine.

7.0 TRAINING

A trainee will conduct the test with supervision initially. Determining egg concentrations and fertilization counts are test specific activities. These functions can be performed independently after a trainee has demonstrated he or she can accurately reproduce the test.

8.0 SAFETY

The sea urchin fertilization toxicity test poses little risk to those performing it. Care should be taken when making and dispensing the 10% buffered formalin solution; use a hood if available, but make sure the test area is well ventilated. Protective gloves can be worn when pipetting or dispensing formalin or potentially toxic samples.

Care should be taken when collecting or otherwise handling sea urchins. Urchin spines are sharp and fragile and may puncture the skin and break off if handled roughly. First aid similar to treatment of wood splinters is effective in this case (removal of spine and treatment with antiseptic). Collection of sea urchins by snorkeling should not be done alone.

9.0 ATTACHMENTS

Attachment 1. Equipment List for Fertilization Toxicity Test
Attachment 2. Pretest to Insure Selection of Quality Gametes
Attachment 3. Water Quality Adjustment Data Form
Attachment 4. Sea Urchin Pretest Data Sheet
Attachment 5. Sea Urchin Pretest Continuation Data Sheet
Attachment 6. Sea Urchin Fertilization/Embryological Development Toxicity Test Gamete Data Sheet
Attachment 7. Sea Urchin Fertilization Toxicity Test Fertilization Data Sheet

10.0 REFERENCES

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Hamilton, M.A., R.C. Russo, and R.V. Thurston. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. Environ. Sci. Technol. 11(7):714-719; Correction 12(4):417 (1978)

Sokal, R.R., and F.J. Rohlf. 1981. Biometry. 2nd edition. W.H. Freeman and Company, San Francisco, CA 859 pp.

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Attachment 1

EQUIPMENT LIST FOR FERTILIZATION TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL KIMBLE scintillation vials (These should be type shipped with caps off, and without cap liners. If other brand or type is used, the vials should be tested for toxicity prior to use.)
400 mL beaker or wide-mouthed thermos for holding vials of sperm
250 mL beakers (4)
Pasteur pipettes and latex bulbs
plastic microcentrifuge tubes
25 mL shell vials or equivalent
Test tube rack (to hold shell vials)
12V transformer with pencil type electrodes
Styrofoam (or something to hold electrode tips)
10 cc syringe with large diameter blunt ended needle (make by grinding sharp point off the needle with a grinding stone)
Marking pens
Ice
10-100 μL pipetter
50-200 μL pipetter
5 mL pipetters (2)
Counting slide such as Sedgewick-Rafter chamber
Compound microscope with 10x objective and dark field capability
Hand tally counter
Calculator
Timer for exposure / incubation periods
Buffered formalin and dispenser
Filtered (0.45 μm) seawater, adjusted to 30 ‰
Data sheets
Baker reagent grade water
Approximately 100 ‰ concentrated brine

Attachment 2
PRETEST TO INSURE SELECTION OF QUALITY GAMETES

1. Using the procedure in section 2.4.1, select 2 to 5 females and at least 2 male urchins to be used in the pretest.

2. Fill pretest vials with five mL of **reference** water. There should be at least two vials for each combination of male, female, and pretest sperm concentration (step 4 below). For example, in a pretest with two females, one male, and six pretest sperm concentrations, 24 vials (2 X 2 X 6) would be needed. Arrange and mark vials accordingly in a rack.

3. Perform steps 2.4.2 (egg collection) and 2.4.3 (egg dilution) for each female urchin. Make enough volume of the egg suspension to perform the pretest and the test.

4. Perform step 2.4.4 (sperm collection) for each male urchin or male combination. Prepare a dilution series of sperm concentrations which will bracket the 60-90% fertilization rate in the test. Sperm dilution will depend on the health and reproductive status of the male urchin, but in most cases the following "standard dilution" should be used:

1: 250 (20 µL dry sperm added to 5 mL MFS. This concentration is used only as stock solution to make up the rest of the dilution series and is not used full strength in the pretest.)
1: 1250 (1 mL of 1:250 and 4 mL MFS)
1: 2500 (1 mL of 1:250 and 9 mL MFS)
1: 5000 (2 mL of 1:2500 and 2 mL MFS)
1: 7500 (2 mL of 1:2500 and 4 mL MFS)
1:10000 (3 mL of 1:7500 and 1 mL MFS)
1:12500 (1 mL of 1:2500 and 4 mL MFS)

Sperm must be used within 30 minutes of dilution. Leave undiluted sperm on ice and retain, because a new sperm dilution of the concentration determined in this pretest will be needed for the toxicity test. **Sperm diluted for use in the pretest may not be used in the toxicity test, because the time elapsed since the addition of water is too great.**

5. As in section 3.0 add 50 µL of the diluted sperm to each pretest vial. Incubate for 30 minutes at approximately 20°C, and add 200 µL of the egg suspension. Incubate for another 30 minutes, then fix with 1 mL of the buffered formalin solution.

6. As in section 4.0, obtain a fertilization rate for the vials. There is no need to count all vials, enough vials should be counted to determine a good male/female combination, and an appropriate sperm dilution factor. If more than one male/female combination is acceptable, this is a good opportunity to choose a female which exhibits easily visible fertilization membranes or in cases where there are many samples, to combine eggs from different females . The appearance of the fertilization membranes may vary among female urchins, and presence of easily visible membranes facilitates counting.

Attachment 3

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL____________________ INITIALS__________

SAMPLE DESIGNATION____________________ DATE__________

A. Salinity Adjustment:

Initial volume (mL)	__________
Initial salinity (‰)	__________
Vol. Milli-Q water added (mL)	__________
Vol. __‰ brine added (mL)	__________
% of original sample (initial vol./final vol. x 100)	__________

B. Character of Sample (after salinity adjustment):

Volume (mL)	__________
Salinity (‰)	__________
pH	__________
Dissolved oxygen (mg/L)	__________
DO saturation (%)	__________
Total ammonia (mg/L)	__________
Sulfide (mg/L)	__________

COMMENTS______________________________________

Attachment 4

SEA URCHIN PRETEST DATA SHEET

TEST ID________________________ INITIALS________________

STUDY PROTOCOL________________________ DATE________________

EGGS

Female number:	____	____	____	____
Collection time:	____	____	____	____
Count:	____	____	____	____

SPERM

Male number:	____	____	____	____
Collection time:	____	____	____	____
Dilution start time:	____	____	____	____

TEST TIMES

Sperm in:__________ Eggs in:__________ Formalin in:____________

SPERM DILUTION ______________________________

COMMENTS ____

____

% FERTILIZATION Reference sample designation:________________

Female # ________________ Male # ____________

Sperm Dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:________________

Female # ________________ Male # ____________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

Attachment 5

SEA URCHIN PRETEST CONTINUATION DATA SHEET

TEST ID____________________ INITIALS__________

STUDY PROTOCOL____________________ DATE__________

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

Attachment 6

SEA URCHIN FERTILIZATION/EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST GAMETE DATA SHEET

TEST ID____________________ INITIALS__________

STUDY PROTOCOL____________________ DATE__________

EGGS

Collection time:____________________

Initial count/volume:____________________

Final count:____________________

SPERM

Collection time:__________ Dilution start time:__________

Sperm dilution:____________________

Test start temperature:____________________

TEST TIMES

Box #	Sperm in:	Eggs in:	Formalin in:

COMMENTS____________________

Attachment 7

SEA URCHIN FERTILIZATION TOXICITY TEST

FERTILIZATION DATA SHEET

TEST ID____________________ INITIALS__________

STUDY PROTOCOL____________________ DATE__________

PERCENT FERTILIZED

Replicate

Treatment	1	2	3	4	5	Mean±SD	Unfert.

COMMENTS ____________________

This is NOT A CITABLE DOCUMENT
and is intended for reference only.

Date Prepared : April 10, 1990

Date Revised: August 15, 1995

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

1.0 OBJECTIVE

The purpose of the embryological development toxicity test with the sea urchin, *Arbacia punctulata*, is to determine if a sea water, pore water, sea surface microlayer, or other sample affects development of exposed embryos (development arrested at an early stage or a developmental abnormality) relative to that of embryos exposed to a reference sample. The test may also be used to determine the concentration of a test substance which affects development. Test results are reported as treatment (or concentration) which produces statistically significant developmental effect. This test can be performed concurrently with Sea Urchin Fertilization Toxicity Test (SOP 10.6) and/or Sea Urchin Genotoxicity/Teratogenicity Test (SOP 10.8), using the same pretest and sperm and egg collection.

2.0 TEST PREPARATION

2.1 Test Animals

Gametes from the sea urchin, *Arbacia punctulata* are used in the sea urchin embryological development toxicity test. Animals can be collected in the field or obtained from a commercial supplier. *A. punctulata* can be differentiated from other species of urchins which are found in Texas by the five plates surrounding the anal opening, and by round sharp spines on the dorsal surface of the test and flattened spines surrounding the Aristotle's lantern. Urchins can be maintained easily in aquaria or other tanks with running seawater or an aquarium filter. Urchins will eat a wide variety of marine vegetation. A good diet may be provided by placing rocks from jetties (which have been colonized by diatoms and macroalgae) into the tank with the urchins or romaine lettuce may be provided as a substitute. Temperature manipulations of the cultures will prolong the useful life of the urchins. Cultures are maintained at $16 \pm 1°C$ when gametes are not required. Temperature is gradually increased to $19 \pm 1°C$ at least one week prior to gamete collection and subsequently decreased if no further tests are planned. Photoperiod is maintained at 16 hours of light per day. Water quality parameters should be monitored weekly and salinity maintained at 30 ± 3 ‰. Males and females should be kept in separate tanks.

2.2 Dilution Water

HPLC reagent grade purified water or concentrated seawater brine is used to adjust samples to 30 ‰ as described in Water Quality Adjustment of Samples (SOP 10.12). Concentrated seawater brine (90-110 ‰) is made in large batches by heating seawater to 40°C or less in large tanks with aeration for 3-4 weeks. Brine quality will remain constant over long periods with no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen are also measured. Salinity adjustment and water quality data are recorded on prepared data forms.

Filtered (0.45 µm) seawater adjusted to 30 ‰ is used to wash eggs and is also used for sperm and egg dilutions. The acronym MFS (for Millipore® filtered seawater) is used for this filtered and salinity adjusted seawater.

2.3 Test System: Equipment

When testing samples for potential toxicity, five replicates per treatment are recommended. One replicate is a 5 mL volume of sample in a disposable glass scintillation vial. When conducting a dilution series test, fifty percent serial dilutions may be made in the test vials, using MFS as the diluent.

2.3.1 Equipment

A list of equipment necessary for conducting this test is given in Attachment 1 (Equipment List for Embryological Development Toxicity Test).

2.3.2 Solutions

<u>10% Buffered Formalin</u>:

1,620 mL sea water
620 mL formaldehyde
6.48 g NaH_2PO_4 or KH_2PO_4 (mono)
10.5 g Na_2HPO_4 or K_2HPO_4 (dibasic)

1 mL needed for each replicate. Fill the dispenser.

2.4 Collection and Preparation of Gametes

Quality gametes must first be collected, and then diluted to the appropriate concentration for addition to the test vials.

2.4.1 Selection of Urchins to be Used in Toxicity Test.

1. Take two or three females and place in shallow bowl, barely covering tests with seawater.

2. Stimulate release of eggs from gonopores of a female by touching test with electrodes from a 12V transformer.

3. Collect a few eggs from between spines using a 10 mL disposable syringe with a large gauge blunt-tipped needle attached. Discard the first small quantity of eggs expelled from each gonopore and continue collecting. Place a 2 to 5 drops of eggs onto a scintillation vial containing 10mL of filtered seawater. Rinse syringe and repeat for each female.

4. Select females which have round, well developed eggs, and which do not release clumps of eggs or undeveloped ovarian tissue.

5. Place 2-4 males in shallow bowl(s) with a small amount of seawater, leaving the upper $^1/_2$ to $^1/_3$ of the animals uncovered.

6. Stimulate release of sperm from gonopores by touching test with electrodes from 12V transformer (about 30 seconds each time). If sperm is watery, reject the animal and choose another. Sperm should be the consistency of condensed milk. Collect sperm using a pastuere pipette with a rubber bulb attached.

Generally, a gamete check is performed in order to ensure that both the male and the female urchins used in the test have gametes with a high degree of viability. If the gamete check is performed, two to five females and at least two males should be selected using the above procedures. The check is performed by adding 5 to 7 drops of a concentrated dilution of sperm to the eggs in the scintillation vials (collected as described above) and observing the eggs under the microscope after 10 minutes. The concentrated dilution of sperm is usually made by diluting 20-50μL of sperm in 10 mL of filtered seawater. If the proportion of eggs fertilized is high (95-100%), that female and male may be used in the pretest and test. Sperm from a number of males or eggs of females may be combined if the gamete check reveals a number of high quality animals or the confidence is high in the quality of the gametes Once a good male and female are selected a pretest can be conducted to determine the correct dilution of sperm to use in the test (Attachment 2).

2.4.2 Obtain Eggs

1. Place selected female in large Carolina dish and add enough water to cover the urchin's test with approximately 1 cm of seawater. Stimulate release of eggs from female with 12V transformer.

2. Collect eggs as above using the 10 mL syringe. Remove needle before dispensing eggs into a disposable shell vial or other clean container capable of holding 25-50 mL. Collect enough eggs for pretest and test. If female stops giving eggs readily or starts giving chunky material, cease stimulation and collection of eggs from that female.

3. Add MFS to fill shell vials, gently mixing eggs. Allow eggs to settle to bottom of vial. Remove water with a pipette. Replace water, again gently mixing the eggs.

4. Repeat washing procedure.

2.4.3 Prepare Appropriate Egg Concentration

1. Put approximately 100 mL of 30 $^{\circ}/_{\infty}$ MFS in a 250 mL beaker, and add enough washed eggs to bring the egg density to approximately 10,000 per mL . If more than 400 total replicates (27 treatments) are to be tested, a larger amount of water and a correspondingly larger amount of eggs should be used. Two hundred µL of this egg solution will be used per replicate, and it is easier to maintain proper mixing and uniform egg density if there is an excess of at least 50%.

2. Check egg density and adjust to within approximately 9000 to 11,000 eggs per mL, as follows. Gently swirl egg solution until evenly mixed. Using a pipette, add 1 mL of the solution to a vial containing nine mL seawater. Mix and transfer 1 mL of this diluted solution to a second vial containing 4 mL of seawater. Again, mix and transfer 1 mL of this diluted solution to a counting slide such as a Sedgewick-Rafter slide.

3. Using a microscope (either a compound microscope with a 10x objective or a dissecting scope may be used here), count the number of eggs on the slide. If the number is not between 180 and 220, then adjust by adding eggs or water. If egg count is > 220 use the following formula to calculate the amount of water to add: -

   ("egg count" - 200/200) x Current Volume of Eggs = Volume seawater to add to stock (mL)

   If egg count < 200 add a small amount of eggs. Since it is less arbitrary and more likely to arrive at an acceptable count when using the water addition formula, it is better to originally overestimate the amount of eggs to add to the 100 mL of water.

4. Repeat steps 2 and 3 until an acceptable egg count (between 180 and 220) is obtained.

5. Just before the eggs are to be used, add 2 mL of a penicillin-G stock solution (5000 units/mL) per 100 mL of eggs in the egg suspension. The addition of penicillin to the embryological development test has been shown to be beneficial in evalution of the stages of development by inhibiting bacterial growth which can cause the embryos to disintegrate before the test is terminated.

   The penicillin stock solution is prepare by diluting 296 mg of Penicillin-G sodium salt (1690 units/mg) in 100 mL of MFS and mixing until dissolved. The addition of 2 mL/100 mL of eggs will result in a final concentration of 4 units/mL in each replicate. The number of units of penicillin per mg of penicillin-G sodium salt is variable with each lot. Thus, the quantity added to the stock will change in order to keep the final concentration at 4 units/mL.

2.4.4 Obtain Sperm

Place selected male urchin in a large Carolina dish containing 1-2 cm of water. About half of test should be above water level. Stimulate male with 12V transformer, and collect about 0.5 mL of unwetted sperm from between spines using a pasteur pipette. Place sperm into a plastic microcentrifuge tube. Keep on ice until used. Be careful not to add any water or sperm which has contacted water to the vials. High quality sperm collected dry and kept on ice will last at least eight hours without measurable decline in viability.

2.4.5 Prepare Appropriate Sperm Dilution

As in the Sea Urchin Fertilization Test, it is desirable for control fertilization to be within 60-90%. Although controls outside these bounds do not automatically disqualify a test, particularly if a valuable dose response is generated, the chance of inducing polyspermy is increased with increased concentrations of sperm, and good dose responses may be difficult to obtain with less than 60% fertilization in controls. Density of sperm in the sperm solution should be determined with this goal in mind. Condition of the animals and length of acclimation to the aquarium may effect the chosen sperm density. The pretest (Attachment 2) may be used to calculate an appropriate sperm dilution. Generally, a dilution of between 1:10,000 and 1:2500 will result in desirable fertilization rates, if the animals are in good condition.

For example, if a sperm dilution of 1:5000 is required (as determined from the pretest), add 20 µL sperm to 10 mL MFS. Mix thoroughly, then add 1 mL of this solution to 9 mL MFS. Sperm should not be wetted until just before starting the test. Sperm wetted more than 30 minutes before the test has begun, including sperm dilutions used in any pretest, should be discarded and a new dilution made from sperm kept on ice.

3.0 TEST PROCEDURES

1. While gently swirling the egg solution to maintain even mixing of eggs, use a 200 μL pipetter to add 200 μL diluted egg suspension to each vial. Record time of egg addition.

2. Add 50 μL appropriately diluted sperm to each vial. Record time of sperm addition. Sperm should be used within 30 minutes of wetting.

3. Incubate all test vials at 20 $\pm$ 1°C for 48 hours.

4. Using the dispenser, add 1 mL 10% buffered formalin to each vial.

5. Vials may now be capped and stored overnight or for several days until evaluated.

4.0 DATA COLLECTION AND TABULATION

1. Transfer approximately 1 mL embryos and water from <u>bottom</u> of test vials to counting slide. Observe embryos using a compound microscope under 100X magnification.

2. Count 100 embryos/sample using hand counter with multiple keys (such as a blood cell counter), using one key to indicate normally developed pluteus larvae and others to indicate unfertilized eggs, embryos arrested in earlier developmental stages, and other abnormalities. Attachment 3 has a list of developmental stages and drawings of each.

3. Calculate the proportion of normal plutei for each replicate test:

$$\frac{\text{Number normal plutei X 100}}{\text{Total no. eggs/embryos}} = \text{Percent normal plutei}$$

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments 4-9). Normally, percent normal development (normal plutei) in each treatment is compared to an appropriate reference treatment (seawater, pore water or sea surface microlayer from an uncontaminated environment). Statistical comparisons are made using analysis of variance (ANOVA) and Dunnett's *t*-test (Sokal and Rohlf 1981) on the arc sine square root transformed data. For multiple comparisons among treatments, Ryan's Q test (Day and Quinn 1989) with the arc sine square root transformed data is recommended. The trimmed Spearman-Karber method

with Abbott's correction is recommended to calculate EC_{50} values for dilution series tests (Hamilton et al. 1977)

6.0 QUALITY CONTROL

Quality control tests may be run using both positive and negative controls with multiple replicates (as many as desired). Typically, a reference toxicant dilution series (sodium dodecyl sulfate) is tested with each test to evaluate the effectiveness of the sperm dilution chosen. Negative controls may include a reference porewater, filtered seawater, and/or a reconstituted brine.

7.0 TRAINING

A trainee will conduct the test with supervision initially. Determining egg concentrations, embryological stages and counts are test specific activities. These functions can be performed independently after a trainee has demonstrated he or she can accurately reproduce the test.

8.0 SAFETY

The sea urchin embryological development toxicity test poses little risk to those performing it. Care should be taken when making and dispensing the 10% buffered formalin solution; use a hood if available, but make sure the test area is well ventilated. Protective gloves can be worn when pipetting or dispensing formalin or potentially toxic samples.

Care should be taken when collecting or otherwise handling sea urchins. Urchin spines are sharp and fragile and may puncture the skin and break off if handled roughly. First aid similar to treatment of wood splinters is effective in this case (removal of spine and treatment with antiseptic). Collection of sea urchins by snorkeling should not be done alone.

9.0 ATTACHMENTS

Attachment 1. Equipment List for Embryological Development Toxicity Test
Attachment 2. Pretest to Insure Selection of Quality Gametes
Attachment 3. Development of Sea Urchin Eggs to Pluteus Larvae
Attachment 4. Water Quality Adjustment Data Form
Attachment 5. Sea Urchin Pretest Data Sheet
Attachment 6. Sea Urchin Pretest Continuation Data Sheet
Attachment 7. Sea Urchin Fertilization/Embryological Development Toxicity Test Gamete Data Sheet
Attachment 8. Sea Urchin Embryological Development Test Data Sheet
Attachment 9. Sea Urchin Embryological Development Test Continuation Data Sheet

10.0 REFERENCES

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Attachment 1

EQUIPMENT LIST FOR EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST

Large Carolina dishes (at least 2)
20 mL KIMBLE scintillation vials (These should be type shipped with caps off, and without cap liners. If other brand or type is used, the vials should be tested for toxicity prior to use.)
400 mL beaker or wide-mouthed thermos for holding vials of sperm
250 mL beakers (4)
Pasteur pipettes and latex bulbs
plastic microcentrifuge tubes
25 mL shell vials or equivalent
Test tube rack (to hold shell vials)
12V transformer with pencil type electrodes
Styrofoam (or something to hold electrode tips)
10 cc syringe with large diameter blunt ended needle (make by grinding sharp point off the needle with a grinding stone)
Marking pens
Ice
10-100 µL pipetter
50-200 µL pipetter
5 mL pipetters (2)
Counting slide such as Sedgewick-Rafter chamber
Compound microscope with 10x objective and dark field capability
Hand tally counter
Calculator
Timer for exposure / incubation periods
Buffered formalin and dispenser
Filtered (0.45 µm) seawater, adjusted to 30 ‰
Data sheets
Baker reagent grade water
Approximately 100 ‰ concentrated brine

Attachment 2
PRETEST TO INSURE SELECTION OF QUALITY GAMETES

1. Using the procedure in section 2.4.1, select 2 to 5 females and at least 2 male urchins to be used in the pretest.

2. Fill pretest vials with five mL of **reference** water. There should be at least two vials for each combination of male, female, and pretest sperm concentration (step 4 below). For example, in a pretest with two females, one male, and six pretest sperm concentrations, 24 vials (2 X 2 X 6) would be needed. Arrange and mark vials accordingly in a rack.

3. Perform steps 2.4.2 (egg collection) and 2.4.3 (egg dilution) for each female urchin. Make enough volume of the egg suspension to perform the pretest and the test.

4. Perform step 2.4.4 (sperm collection) for each male urchin or male combination. Prepare a dilution series of sperm concentrations which will bracket the 60-90% fertilization rate in the test. Sperm dilution will depend on the health and reproductive status of the male urchin, but in most cases the following "standard dilution" should be used:

1:250 (20 μL dry sperm added to 5 mL MFS. This concentration is used only as stock solution to make up the rest of the dilution series and is not used full strength in the pretest.)
1: 1250 (1 mL of 1:250 and 4 mL MFS)
1: 2500 (1 mL of 1:250 and 9 mL MFS)
1: 5000 (2 mL of 1:2500 and 2 mL MFS)
1: 7500 (2 mL of 1:2500 and 4 mL MFS)
1:10000 (3 mL of 1:7500 and 1 mL MFS)
1:12500 (1 mL of 1:2500 and 4 mL MFS)

Sperm must be used within 30 minutes of dilution. Leave undiluted sperm on ice and retain, because a new sperm dilution of the concentration determined in this pretest will be needed for the toxicity test. **Sperm diluted for use in the pretest may not be used in the toxicity test, because the time elapsed since the addition of water is too great.**

5. As in section 3.0 add 50 μL of the diluted sperm to each pretest vial. Incubate for 30 minutes at approximately 20°C, and add 200 μL of the egg suspension. Incubate for another 30 minutes, then fix with 1 mL of the buffered formalin solution.

6. As in section 4.0, obtain a fertilization rate for the vials. There is no need to count all vials, enough vials should be counted to determine a good male/female combination, and an appropriate sperm dilution factor. If more than one male/female combination is acceptable, this is a good opportunity to choose a female which exhibits easily visible fertilization membranes or in cases where there are many samples, to combine eggs from different females . The appearance of the fertilization membranes may vary among female urchins, and presence of easily visible membranes facilitates counting.

Attachment 3

DEVELOPMENT OF SEA URCHIN EGGS TO PLUTEUS LARVAE

The development of sea urchin eggs from fertilization to pluteus larvae normally occurs in approximately 48 hours. Although development is a continuous process of mitosis and cellular differentiation, developmental biology defines distinct stages of development by gross morphological characteristics. For the purpose of the Sea Urchin Embryological Development Test, six stages are defined and used in the characterization of embryos (Drawings on following page).

1. Unfertilized egg - single cell which appears dense and lacks a fertilization membrane.

2. Fertilized egg - egg with a distinct fertilization membrane which appears as a thin band lying slightly away from the central egg. The early stages of cell division are included in this group.

3. Blastula - spherical, "hollow-ball" stage which is ciliated and becomes free-swimming by breaking out of the fertilization membrane.

4. Early gastrula - beginnings of invagination of the blastula wall are evident. Cells move inward (invaginate) to form a central cavity (archenteron). Early gastrula includes embryos with the earliest stages of invagination and continues until the archenteron reaches approximately two-thirds of the diameter of the embryo.

5. Late gastrula - gastrula in which archenteron has developed in length to two-thirds of the embryo diameter and has begun to differentiate and bend towards and break through the embryo wall. Included are the later stages (prism) with primitive gut (complete digestive system), early skeletal rod development, and beginnings of deltoid shape formation.

6. Pluteus - deltoid-shaped larval stage with complete digestive system, skeletal rods, and growth of projecting arms.

Attachment 4

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL____________________ INITIALS__________

SAMPLE DESIGNATION____________________ DATE__________

A. Salinity Adjustment:

Initial volume (mL)	__________
Initial salinity (‰)	__________
Vol. Milli-Q water added (mL)	__________
Vol. __‰ brine added (mL)	__________
% of original sample (initial vol./final vol. x 100)	__________

B. Character of Sample (after salinity adjustment):

Volume (mL)	__________
Salinity (‰)	__________
pH	__________
Dissolved oxygen (mg/L)	__________
DO saturation (%)	__________
Total ammonia (mg/L)	__________
Sulfide (mg/L)	__________

COMMENTS____

Attachment 5

SEA URCHIN PRETEST DATA SHEET

TEST ID____________________ INITIALS____________________

STUDY PROTOCOL____________________ DATE____________________

EGGS

Female number:	____	____	____	____
Collection time:	____	____	____	____
Count:	____	____	____	____

SPERM

Male number:	____	____	____	____
Collection time:	____	____	____	____
Dilution start time:	____	____	____	____

TEST TIMES

Sperm in:________ Eggs in:________ Formalin in:________

SPERM DILUTION ____________________

COMMENTS ____________________

____

% FERTILIZATION Reference sample designation:____________

Female #________ Male #________

Sperm Dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

% FERTILIZATION Reference sample designation:____________

Female #________ Male #________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
____	____	____	____	____
____	____	____	____	____
____	____	____	____	____

Attachment 6

SEA URCHIN PRETEST CONTINUATION DATA SHEET

TEST ID____________________ INITIALS__________

STUDY PROTOCOL____________________ DATE__________

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
___	___	___	___	___
___	___	___	___	___
___	___	___	___	___
___	___	___	___	___

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
___	___	___	___	___
___	___	___	___	___
___	___	___	___	___
___	___	___	___	___

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
___	___	___	___	___
___	___	___	___	___
___	___	___	___	___
___	___	___	___	___

% FERTILIZATION Reference sample designation:__________

Female #__________ Male #__________

Sperm dilution	REP 1	REP 2	REP 3	REP 4
___	___	___	___	___
___	___	___	___	___
___	___	___	___	___

Attachment 7

SEA URCHIN FERTILIZATION/EMBRYOLOGICAL DEVELOPMENT TOXICITY TEST GAMETE DATA SHEET

TEST ID________________________ INITIALS______________

STUDY PROTOCOL____________________ DATE______________

EGGS

Collection time:______________________________

Initial count/volume:__________________________

Final count:__________________________________

SPERM

Collection time:______________ Dilution start time:______________

Sperm dilution:____

____

Test start temperature:_________________________________

TEST TIMES

Box #	Sperm in:	Eggs in:	Formalin in:
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______
____	______	______	______

COMMENTS____

____

____

Attachment 8

SEA URCHIN EMBRYOLOGICAL DEVELOPMENT TEST DATA SHEET

TEST ID____________________ INITIALS____________________

STUDY PROTOCOL____________________ DATE____________________

Test Start (date & hour)________________ Test stopped (date & hour)________________

Treatment	Rep.	Eggs	Blastula	Early Gastrula	Late Gastrula	Pluteus	% Normal Development	%Non-Norm

COMMENTS____

Attachment 9

EMBRYOLOGICAL DEVELOPMENT TEST CONTINUATION DATA SHEET

Test Id____________________ Initials ____________________

Study protocol____________________ Date____________________

Treatment	Rep.	Eggs	Blastula	Early Gastrula	Late Gastrula	Pluteus	% Normal Development	%Non-Norm

COMMENTS____

Date Prepared : 4-10-90

Date Revised : 7-8-97

DINOPHILUS GYROCILIATUS TOXICITY TEST

1.0 OBJECTIVE

The purpose of the *Dinophilus gyrociliatus* toxicity test is to determine if a sea water, pore water, sea surface microlayer, or other sample reduces survival and/or reproduction in exposed *D. gyrociliatus* polychaetes relative to those exposed to a reference sample. The test may also be used to determine the concentration of a test substance which reduces survival or reproduction. Test results are reported as treatment (or concentration) which produces statistically significant reduced survival or reproduction.

2.0 TEST PREPARATION

2.1 Test Animals

Recently hatched juvenile *D. gyrociliatus* are needed to perform this test. These polychaetes are very easy to culture in the lab. Seed animals for a culture can be collected in the field.

Cultures can be maintained easily in 25-30 $^{\circ}/_{\circ\circ}$ seawater in small widemouth jars or almost any tightly closable container. Cultures are fed a suspension of freeze-dried powdered (<105 µm) spinach every 1-2 weeks. Cultures are generally reestablished every month by transferring a portion of an existing culture into a new culture vessel and adding fresh seawater to make up the difference in the volume. New cultures produce the greatest number of juveniles for use in testing, however cultures may be maintained for several months to provide seed stocks for new cultures. The salinity of cultures should be checked weekly and recorded on standardized data forms (Attachment 1).

2.2 Procurement of Test Organisms From the Cultures

Choose a culture container which has had sufficient time since it was established to produce a sufficient number of juveniles for harvesting (usually about 2-3 weeks). Place a light source such as a fiber optic light at the edge of the jar, near the surface of the water. The newly released juveniles are positively phototactic and will congregate near the light. Using a pasteur pipet and a dissecting microscope, move the animals from the jar into a smaller dish containing fresh

filtered seawater. Salinity of the test water should be similar to culture conditions to prevent osmotic shock to the animals.

2.3 Dilution Water

Milli-Q water or concentrated seawater brine is used to adjust samples to the proper salinity (Attachment 2). Concentrated seawater brine (90-110 ‰) is made in large batches by heating seawater to 40°C or less in large tanks with aeration for 3-4 weeks. Brine stock quality remains constant over long periods with no refrigeration. At the time of salinity adjustment, pH, ammonia, and dissolved oxygen is also measured. Salinity adjustment and water quality data is recorded on prepared data forms.

2.4 Test System: Equipment

A list of equipment necessary for conducting this test is given in Attachment 3 (Equipment List for *Dinophilus gyrociliatus* Toxicity Test).

3.0 TEST PROCEDURES

3.1 Experimental Design

The tests are conducted in 20-mL stender dishes with ground glass lids with 10 mL of solution per dish. At least four animals are placed into each dish with five dishes per treatment. If brine and Mill-Q water are used as diluents, then both diluted brine and natural seawater controls can be run, as well as an appropriate reference sample. Tests may be conducted as a screening test (one treatment concentration) or as a dilution series test (more than one treatment concentration). The test is run as a static exposure with no water change during the test period.

3.2 Test Initiation

The test is started with one- to two-day-old animals. An experienced investigator can easily differentiate between newly released juveniles and more mature animals due to their rapid growth. The test solutions are first dispensed to the exposure chambers. The animals are taken from the small dish described in Section 2.2 and placed individually into the chambers using a Pasteur pipet with a latex bulb. All observations and manipulations are performed using a dissecting microscope. After the animals have been added, each chamber is reexamined to

verify that there are at least four animals per replicate at the start of the test. After the chambers have been reexamined, 50 µL of a 0.5 percent powdered spinach solution is dispensed to each dish.

4.0 DATA COLLECTION

4.1 Record Keeping

All raw data are entered on standardized forms (Attachments 4 and 5). Raw data sheets are kept on file in the lab, and a copy made and kept on file in the care of Project Leader.

4.2 Biological Monitoring

Each chamber is examined at 24 hours (Day 1), 96 hours (Day 4), and at test completion (Day 7). Survival and reproductive data for each chamber are recorded on a standardized data sheet (see Attachment 3). The eggs of *Dinophilus gyrociliatus* are sexually dimorphic with the female eggs being much larger than the males. There are generally 2 to 5 eggs/egg case with the majority of the eggs being female. Because the males die shortly after copulation, which occurs in the egg case, only female eggs are used in the egg production counts. The first eggs are usually laid on Day 4 or 5. New juveniles may begin to emerge by Day 6 or 7. The reproductive data recorded for each chamber are the total number of female eggs, the number of egg cases, the number of eggs still in the coelom, and the number of newly emerged juveniles.

4.3 Environmental Monitoring

The parameters of temperature, salinity, dissolved oxygen, pH, and ammonia concentration will be made on a composite sample of the test solution for each treatment just prior to test initiation and again on Day 7 at the time of test completion. The data will be recorded on the Environmental Conditions Data Form (Attachment 4).

The water quality parameters for the static tests should be maintained within the following ranges:

Parameter	Acceptable Range
Temperature	20°C ± 2°C
Salinity	Test specific ± 2 ‰
Dissolved oxygen	≥ 60% Saturation
pH	7.9 ± 0.4 units

5.0 DATA ANALYSIS

Data are recorded on standardized data sheets (See Attachments 1, 2, 4, and 5). Normally, survival and/or reproduction in each treatment is compared to an appropriate reference treatment (seawater, pore water or sea-surface microlayer sample from an uncontaminated environment). Statistical comparisons are made using analysis of variance (ANOVA) and Dunnett's test (Sokal and Rohlf 1981). Since ANOVA assumes that responses are independently and normally distributed with a common variance within treatment levels, a test of the validity of these assumptions is recommended. Bartlett's test or Levine's test may be used to test for homogeneity of variances (Snedecor and Cochran 1980). If the raw data do not satisfy these assumptions, the data may be transformed (for example a natural log or a $\log_{10}$ transformation) to stabilize the variance between treatment levels. If the assumptions for ANOVA cannot be met, a non-parametric Kruskal-Wallis test (Daniel 1978) may be performed.

The trimmed Spearman-Karber method with Abbott's correction is recommended to calculate EC_{50} values for dilution series tests (Hamilton et al. 1977)

6.0 QUALITY CONTROL

Reconstituted brine, fresh filtered seawater, and reference site controls may be run. A test is unacceptable if more than 20% of control organisms appear stressed or diseased, or die.

7.0 TRAINING

A trainee will conduct the test with supervision initially. Determining egg concentrations and fertilization counts are test specific activities. These functions can be performed independently after a trainee has demonstrated he or she can accurately reproduce the test.

8.0 SAFETY

The *Dinophilus gyrociliatus* toxicity test poses little risk to those performing it. Protective gloves and lab coats should be worn when pipetting or dispensing potentially toxic samples.

9.0 ATTACHMENTS

Attachment 1. Culture Maintenance Record
Attachment 2. Water Quality Adjustment Form
Attachment 3. Equipment List for *Dinophilus gyrociliatus* Toxicity Test
Attachment 4. Toxicity Test Environmental Conditions
Attachment 5. Biological Monitoring Data

10.0 REFERENCES

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Attachment 1

CULTURE MAINTENANCE RECORD

Organism: *Dinophilus gyrociliatus* ____ Culture Designation ________________

Date Culture Started: ________________ Project Number ____________________

Date Culture Terminated: ____________

Date/Time	Salinity (‰)	Temp (°C)	Comments	FED Initials

Attachment 2

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL________________________ INITIALS__________

SAMPLE DESIGNATION____________________ DATE__________

A. Salinity Adjustment:

Initial volume (mL) __________

Initial salinity (‰) __________

Vol. Milli-Q water added (mL) __________

Vol. __‰ brine added (mL) __________

% of original sample (initial vol./final vol. x 100) __________

B. Character of Sample (after salinity adjustment):

Volume (mL) __________

Salinity (‰) __________

pH __________

Dissolved oxygen (mg/L) __________

DO saturation (%) __________

Total ammonia (mg/L) __________

Sulfide (mg/L) __________

COMMENTS____

Attachment 3

EQUIPMENT LIST FOR *DINOPHILUS GYROCILIATUS* TOXICITY TEST

Glass stender dishes with ground glass lids (approximately 20-mL size)
Dissecting microscope with illuminator (fiber optics is suggested)
Pasteur pipets (with latex bulbs)
5-mL Oxford-type pipetter (with tips)
50 μL pipetter
2 to 3 small Carolina type dishes
Filtering apparatus (with 0.45-μm filters)
Vacuum pump
Colored labeling tape
Pens and markers
Data sheets
Manual counter
Kimwipes

For Food Preparation:

Freeze-dried spinach (from frozen grocery bought pack)
150-μm sieve
Mortar and pestle or electric coffee grinder

Attachment 4

DINOPHILUS GYROCILIATUS
TOXICITY TEST ENVIRONMENTAL CONDITIONS

Test Material________________________ Test Description__________________________
Date/Time Test Started_______________ Date/Time Test Completed_______________
Observation Period__________________ Date_______________ Time_____________

			Parameter			
					Ammonia (mg/L)	
Treatment	Temp (oC)	Salinity ()	DO (mg/L)	pH	mV	(Mg/L)

Method: ____

Entered by:___________ Date: __________

Observation Period: _______________ Date: __________________ Time: _________

			Parameter			
				Ammonia (mg/L)		
Treatment	Temp (oC)	Salinity ()	DO (mg/L)	pH	mV	(Mg/L)

____ Method:

____

Entered by:___________ Date: __________

Attachment 5

DINOPHILUS GYROCILIATUS

BIOLOGICAL MONITORING DATA

Test Material____________________ Test Description____________________

Date Test Started____________________ Date Test Completed____________________

	Survival Data			Reproductive Data					
				Day 4	Day 7				
Treatment/ Replicate	No. Observed Day 1	No. Observed Day 4	No. Observed Day 7	Total No. Eggs	Total No. Eggs	No. Egg Cases	No. Eggs In Coelom	No. Eggs/ Adult	Comments

Organism Source: ____________________

Data Entered By: ____________________ Approval: ____________________

Comments:____________________

Date Prepared: January 10, 1993

PREPARATION OF FILTERED (0.45 μm) SEAWATER

1.0 APPLICATION

Filtered (0.45 μm) seawater (MFS) is used in most of the toxicity tests conducted at this field station with a variety of marine organisms. The acronym MFS is derived from "Millipore® Filtered Seawater" because the original 0.45 μm filtering apparatus purchased at this lab was manufactured by Millipore company. Filters and apparatus manufactured by other companies are acceptable. MFS is distinct from FS, which indicates seawater of any salinity filtered through a 1 μm cartridge filter. MFS serves an important role in the tests as a nontoxic seawater medium. Among other functions, MFS is used as a control medium, to dilute porewater samples, to wash sea urchin eggs, to dilute sea urchin eggs and sperm, and to overlay sediment in amphipod exposure chambers.

2.0 PREPARATION

2.1 Equipment and Labware

See the Equipment List for Preparation of Filtered (0.45 μm) Seawater (MFS) in Attachment 1.

2.2 Source of Seawater

The seawater to be used in the preparation of MFS is natural and free of contaminants. It is typically pre-filtered using a 1 μm cartridge filter to reduce the quantity of 0.45 μm filters needed. Since the salinity of MFS is 30 $^o/_{oo}$, it is preferable to start with seawater of 30 $^o/_{oo}$. If necessary, adjust seawater salinity to 30 $^o/_{oo}$ as described in Water Quality Adjustment of Samples (SOP F10.12).

3.0 PROCEDURES

1. Set up filtering apparatus (Figure 1). Connect tubing to filtering flask, liquid trap, vacuum pump and valve. Plug in the pump. Secure liquid trap to burette stand with clamp. Place bottom of filtering funnel on filtering flask. Remove one 0.45 μm filter from package with forceps (filters are packaged with a paper liner on both sides), wet the filter, and place on the fritted disc of the filtering funnel. Clamp the top of the filtering funnel into place.

2. Add seawater to the filtering funnel. Close the relief valve. Turn on the pump. Add more seawater as the volume in the funnel drops. Continue until the flow slows noticeably or until the filtering flask becomes full.

3. If the flow slows noticeably, replace the filter. Open the relief valve and turn off the pump in that order. Always open the relief valve before turning off the pump. (Doing otherwise leaves a vacuum inside the pump which could damage it.) Remove the filtering funnel and clamp. Remove the used filter and put on a new filter with the forceps. Return the top of the filtering funnel into place and repeat step 2.

4. If the filtering flask becomes full, transfer the MFS to a plastic holding container of appropriate size. Open the valve and turn off the pump. Remove the filtering funnel and flask. Transfer the MFS in the flask to the holding container, using a funnel if necessary.

5. Continue filtering and transfering until sufficient MFS is prepared.

6. After use, disconnect the pump, tubing and glassware. Rinse the glassware with deionized water.

7. Aerate the MFS. Because the filtering process strips oxygen from seawater, the MFS should be aerated to bring the dissolved oxygen (DO) concentration above 80% saturation. Connect airline tubing to an aquarium pump and to a new disposable glass pipette. Place the pipette into the MFS container and aerate until DO concentration is adequate (measure DO with a dissolved oxygen meter).

8. Double-check salinity of the MFS and adjust as needed.

9. Discard MFS approximately one week after preparation, unless the test to be conducted has different requirements.

4.0 TRAINING

Personnel who perform this task will first read this protocol and then operate under supervision during at least his/her first MFS preparation.

5.0 SAFETY

No safety hazards are known to exist.

6.0 ATTACHMENTS

Attachment 1. Equipment List for Preparation of Filtered (0.45 μm) Seawater (MFS)

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Approved by:

R. Scott Carr
Field Station Leader

Anne E. Kinsinger
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Joseph B. Hunn

Quality Assurance Officer

Attachment 1

EQUIPMENT LIST
FOR PREPARATION OF FILTERED (0.45 μm) SEAWATER (MFS)

Filters (0.45 μm, preferably gridded, Millipore® or other equivalent brand)
Filter forceps
Filter funnel with clamp
Filtering flask (2 L)
Vacuum trap
Small, plastic valve for vaccuum release
Burette stand with clamp
Standard funnel
Beakers (1 L)
Clear vinyl tubing (eg. Tygon®)
Airline tubing (plastic)
Disposable glass pipettes
Air pump (aquarium type)
Refractometer
Dissolved oxygen meter
Containers for holding MFS (polyethylene, high or low density, are good but should be soaked for at least three days with multiple water changes prior to use)

Date Prepared: November 4, 1996

ALGAL ZOOSPORE GERMINATION AND GERMLING GROWTH TOXICITY TEST PROTOCOL

1.0 OBJECTIVE

The purpose of the algal germination and germling growth toxicity test using *Ulva fasciata* and *U. lactuca* zoospores is to determine if sea water, pore water, or other aqueous samples inhibit germination and/or suppress growth of exposed algal zoospores and developing germlings relative to the response of zoospores and germlings exposed to a reference sample.

In this procedure, motile, quadriflagellate zoospores are exposed to test solutions for 96 hours, during which time they settle on glass cover slides in the test chambers. Each slide is examined microscopically to determine the percentage of zoospores that failed to germinate. Also, the length and cell number of ten randomly selected germlings are measured and counted, respectively, for each replicate. Test results are reported as the treatment (or concentration) that produces a statistically significant reduction in germination and growth or as the concentration that reduces germination by 50 percent (EC_{50}).

2.0 TEST SYSTEM

2.1 Equipment

A complete list of equipment necessary to conduct an algal zoospore test is provided in Attachment 1.

2.2 Dilution Water

Ultra-pure or concentrated seawater brine is used to adjust samples and filtered sea water to 30‰ as described in Water Quality Adjustment of Samples (SOP 10.12).

Filtered (0.45µm) seawater adjusted to 30‰ is used to rinse algal samples after collection and rewet thalli to initiate the release of reproductive bodies. It is also used to prepare zoospores stock solutions.

Filtered (0.45µm) seawater adjusted to 30‰ and diluted 10-15% with pore water (also adjusted to 30‰) is used as sample dilution water (DPW). The pore water, which is

extracted from sediment collected from a site known to be free of contamination, provides nutrients necessary for normal algal growth. The amount of pore water added to dilute filtered seawater is pre-determined with a pore water dilution test.

2.3 Test Chambers

Porewater samples may be tested in 20 mL glass beakers *(other containers may be suitable e.g., Stender dishes)*. Place circular (20 mm diameter), glass cover slides flat on the bottom of the test chambers to provide a settling substrate. Five replicates per treatment are recommended. One treatment consists of 10 mL of test solution in a test chamber. When conducting dilution series tests, fifty percent serial dilutions may be made in the test chambers using DPW as the diluent.

3.0 TEST ORGANISMS

3.1 Life History

The test organisms for this protocol are the zoospores of *Ulva fasciata* Delile and *U. lactuca* Linnaeus, two marine, macrophytic Chlorophytes commonly known as sea lettuce. *Ulva* provides food and habitat to vertebrate and invertebrate species.

Ulva fasciata and *U. lactuca* have an alternation of isomorphic gametophytic and sporophytic generations. Motile gametes and zoospores are the primary dispersal mechanism for *Ulva* and are particularly sensitive stages in the life cycle. Each cell in gametophyte and sporophyte blades has the potential to produce 8 to 16 and 4 to 8 reproductive cells, respectively. Gametes and zoospores are differentiated by the number of flagella they possess. Gametes are biflagellate and zoospores are quadriflagellate. Mature sporophytes (2n) release zoospores which settle, germinate and develop into gametophytes (n). Gametophytes reach maturity within six weeks and release gametes which unite and develop into sporophytes, completing the life cycle (Kapraun 1970).

3.2 Species Identification

Both *Ulva fasciata* and *U. lactuca* occur in the intertidal zone. They are common on jetties, bulkheads and other hard substrates and may be found attached to rocks and shells. The two species may be distinguished by thallus morphology. *Ulva fasciata* thalli are divided into narrow, linear segments usually less than 1.5 cm wide but may range from 0.5-5.0 cm wide. *Ulva lactuca* have simple broad thalli with irregular lobes. Consult Kapraun (1970) for more information on *Ulva* sp. in the vicinity of Port Aransas, TX .

3.3 Collection of Algae

Because *Ulva* sp. gametophytes and sporophytes are isomorphic, it is not possible to distinguish one from the other in the field. Positive identification can be made only after reproductive cells have been released.

1. Collect algae at low tide on the evening before a test is to be conducted. During low tide, *Ulva* is exposed to air and becomes slightly desiccated, which is a necessary stage in the zoospore release process. Collect entire plants including the holdfast. The plants collected should be damp; do not collect dry, brittle algae. Place algae in a plastic bucket for transport to the laboratory.

2. Collect at least 20 individual plants from several locations along the jetty. Collections should be made in areas free of pollution to minimize the possibility of genetic or physiological adaptation to pollutants. Samples are collected from several different areas to increase the probability of having several sporophytes among the samples collected.

3. Only collect algae whose thalli are uniform in color or have slightly darker green margins. Algae whose thalli have clear margins should not be collected. Clear margins indicate that reproductive bodies have been released.

3.4 Storage of Algae

1. After collection, rinse samples with filtered (0.45μm) seawater and gently wipe with cheese cloth to remove debris, epiphytes and other associated organisms. Special attention should be given to cleaning the holdfast. The rinsing process should be done as quickly as possible as over-washing may stimulate the algae to release their reproductive bodies prematurely.

2. Discard any small thalli pieces not attached to a holdfast.

3. Layer washed samples (lasagna style, without overlap) between paper towels dampened with filtered (0.45 μm) seawater, place into a box with a lid and keep in the dark at 20°C overnight. Samples should be used within 18 hours of collection.

3.5 Collection of Zoospores

To induce zoospore/gamete release, thalli must be subjected to mild desiccation in the dark, followed by rewetting and a sudden change in light intensity (Reed *et al.* 1991, Anderson and Hunt 1993). Test solutions may be prepared while reproductive bodies are being released.

3.5.1 Zoospore Release

1. Remove several (5-10) clean plants from the dark box. If possible, select plants with dark green or olive colored thalli margins.

2. Place thalli from single plants into 150 or 250 -mL beakers (1 plant/beaker) containing approximately 100 mL of filtered (0.45µm) seawater at 20°C and illuminate with ambient room light (cool white fluorescent).

If thalli from a chosen plant have particularly wide, darkened edges, indicating that a large number of reproductive bodies are available for release, then only two or three thalli and not the entire plant are needed for the release procedure. Place the unused portion of the plant between damp paper towels in a labeled box. If that particular plant is identified as a sporophyte and more zoospores are required for a test, the unused portion will be available. Reproductive bodies should not be collected from plants whose thalli margins have turned tan, brown or golden brown.

3.5.2 Zoospore Identification/Motility Check

Either the formation of a green ring at the water-air interface along the inside of the beaker, or a green cloudiness in the water indicates that reproductive bodies have been released.

1. Examine a sample of the released organisms microscopically (200X) to identify them as zoospores or gametes. Preferably, zoospores from three or four plants should be examined.

2. Once zoospores from several plants have been identified, they should be examined to determine motility. If zoospores from a particular plant are inactive immediately after release, they should not be used in a test and spores from a separate plant should be evaluated. If zoospores are active, they may be accepted as potential test organisms.

3.6 Zoospore Concentration

3.6.1 Concentration Determination

1. Remove thalli from release beaker.

2. Thoroughly mix zoospore solution by stirring and pipet 4.5 mL of the solution into a scintillation vial. Add 0.5 mL of buffered formalin to the scintillation vial.

3. Determine the concentration of the zoospore stock solution subsample microscopically with an Improved Neubauer hemacytometer at 100X.

4. Use the formula and worksheet (Attachment 2) modified from Anderson and Hunt (1993) to calculate the zoospore concentration and the volume of stock solution to add to each test chamber to achieve a 12,750 zoospores/ml concentration. To prevent over-dilution of the test solution, the volume of zoospores added to each test chamber should be between 0.05 and 1% of the test solution volume (i.e., 50 to 100 µl).

5. If the zoospore concentration of the release beaker falls within the specified range to produce 12,750 zoospores/mL of sample, then the release beaker may be used to stock test chambers.

3.6.2 Concentration Adjustments

The concentration of the zoospore stock solution may be adjusted if it is too concentrated or diluted to meet the specified volume range that may be introduced into test solutions.

1. If the zoospore stock solution is too concentrated, dilute it with filtered seawater and recalculate the zoospore concentration.

2. If the stock solution is too dilute, allow zoospores to accumulate at the water-air interface in the release beaker and pipet them into a small beaker. If necessary, water from the bottom of the prepared stock solution may be removed after allowing the zoospores to accumulate at the water's surface. Recalculate the zoospore concentration.

4.0 TOXICITY TEST PROCEDURE

4.1 Exposure to Test Solutions

1. Observe a sample of zoospores from the stock solution before adding them to the test chambers to verify that they are swimming.

2. Pipet the calculated volume of zoospore stock solution into each test chamber.

3. Record the time zoospores are introduced into test chambers on the Algal Test Data Form (Attachment 2).

4.2 Incubation

1. Cover stocked test chambers with clear plastic Petri dish halves (50 mm diameter).

2. Incubate test for 96 h on a 12 h light-12 h dark photoperiod at 20°C.

3. Record the time test chambers are placed into incubators on the Algal Test Data Form (Attachment 2). Zoospores begin to germinate within 48 h. The additional 48 hours allows germling length and cell number to be included as sublethal endpoints.

4.3 Data Collection

The test is terminated after 96 hours. The endpoints for this test are percent germination, germling blade length and germling blade cell number. Salinity from at least five test chambers should be measured and recorded to insure it remained constant throughout the test.

4.3.1 Germination

A zoospore is considered germinated if it has divided into at least two cells; one cell being the initial rhizoid cell which produces a uniserate filament or germ tube, and the other being the frond or blade cell which will give rise to the thallus (Kapraun 1970). However, at 96 hours, germinated zoospores have generally developed into germlings with at least a three or four blade cells. Settled zoospores that have not germinated are usually spherical, between 7 and 10 μm in diameter, and appear light green. Germlings 96 h old are easily differentiated from ciliates or other protists which may be in water samples or may be introduced with the algal zoospores. If an object cannot be identified definitively as a germinated or non-germinated zoospore, it should not be counted.

1. Remove the slide from the test solution and hold it vertically for a moment to allow any test solution to drip off.

2. Invert the cover slide and, using a paper wipe, lightly press it onto a standard microscope slide. Care should be taken when pressing the cover slide onto the microscope slide. If it is pressed too hard, germlings may be destroyed to the point that germling length and cell number data may be impossible to obtain.

3. If necessary, blot around the edge of the cover slide to prevent it from sliding on the microscope slide.

4. Observe the slide microscopically (200X) and record the developmental progress of the first 100 settled zoospores encountered. Record all data on a standardized data sheet (Attachment 3).

4.3.2 Growth measurements

Growth of germlings is determined by measuring the length and counting the number of cells in ten randomly selected germling blades per replicate of each treatment.

1. Randomly select germlings (10) by moving the slide to a new field of view without looking through the eyepiece.

2. With the ocular micrometer, measure the germling lying closest to the micrometer in each field of view and count its cell number. Do not include the rhizoid in germling length measurements. Germling length is initially recorded in ocular units and must be converted to micrometers. (For our Zeiss compound microscope using the 20X objective, the conversion factor is 2.57.)

$$\text{Ocular Units} * 2.57 = \text{germling length } (\mu m)$$

3. If germination is significantly inhibited and fewer than 30% of the zoospores germinate, the first ten germinated zoospores encountered should be measured and counted (Anderson and Hunt, 1993). Record all data on a standardized data sheet (Attachment 3).

4.4 Preservation of Tests

Tests may be preserved by adding 1 mL of 10% buffered formalin to each test chamber. (Preliminary results indicate that there is no significant difference for germling length and cell number between chambers evaluated immediately after test termination and those preserved with formalin and evaluated one week after test termination. The use of gluteraldehyde will be evaluated in the future)

5.0 DATA ANALYSIS

5.1 Statistical Analysis

Percent germination, germling length and germling cell number for each treatment are compared to an appropriate reference.

5.1.1 Germination Data

Statistical comparisons are made using one-way analysis of variance (ANOVA) and Dunnett's *t*-test on arcsine transformed germination data (SAS Institute, Inc 1989). Prior to analysis, transformed data sets should be screened for outliers (SAS Institute, Inc 1992). After removing outliers, data sets should be tested for normality and homogeneity of variance with Levene's test (SAS Institute, Inc 1992).

The trimmed Spearman-Karber method (Hamilton *et al.* 1977) with Abbott's correction (Morgan 1992) is used on germination data to determine the Median Effective Concentration (EC_{50}).

5.1.2 Growth Data

ANOVA and Dunnett's *t*-test are used to determine significant differences of germling length and cell number between test and control treatments. Data sets should be screened for outliers and tested for normality and homogeneity of variance. Appropriate transformations should be applied to germling length and cell number data when assumptions of equal variance are violated.

6.0 QUALITY CONTROL

Quality control tests may be conducted using both positive and negative controls with multiple replicates. Typically, a reference toxicant dilution series (sodium dodecyl sulfate) is included with each test to evaluate the sensitivity of the zoospores chosen. Negative controls may include a reference pore water, dilution water and/or a reconstituted brine.

7.0 TRAINING

A trainee will conduct the test with supervision initially. Determining the zoospore stock solution concentration is a test specific activity. This function can be performed independently after a trainee has demonstrated the ability to accurately reproduce the test.

8.0 SAFETY

The algal zoospore germination and germling growth test poses little risk to those conducting it. Protective gloves may be worn when pipetting potentially toxic samples.

Care should be taken when collecting algae on the jetties. Protective footwear with soles that provide good traction should be worn to protect feet from barnacle cuts and slipping on algal mats. Preferably, collections should not be made alone.

9.0 ATTACHMENTS

Attachment 1. Equipment list for Algal Zoospore Germination and Germling Growth Toxicity Test
Attachment 2. Water Quality Adjustment Data Form
Attachment 3. Zoospore Release Data Form
Attachment 4. Algal Toxicity Test Data Sheet

10.0 REFERENCES

Anderson, B.S. and J.W. Hunt. 1993. Giant kelp germination and growth short-term toxicity test protocol. Procedures Manual for Conducting Toxicity Tests Developed by the Marine Bioassay Project, 90-10WQ.

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Attachment 1

EQUIPMENT LIST FOR ALGAL TOXICITY TEST

20 mL glass beakers or 25 mL plastic beakers for use as test chambers
22 mm diameter circular microscope cover slides and standard microscope slides
50 mm diameter Petri dish halves (or equivalent)
150 or 250 mL glass beakers to conduct zoospore release procedure
1000 mL glass beaker for dilution water preparation
25 mL and 100 mL graduated cylinder
Pasteur pipets and latex bulbs
Improved Neubauer Hemocytometer
Compound microscope with ocular micrometer and 10X and 20X objectives
Thermometer
Refractometer
Writing pens
50-100 µl pipetter
5 mL pipetter
Hand tally counter
Standard, glass microscope slides
Calculator
Plastic bucket to collect algae from the jetties
Filtered sea water (0.45 µm), adjusted to 30‰
Filtered sea water (0.45 µm), adjusted to 30‰ with pore water added
Concentrated brine
Ultra-pure water
Algae Test Data Form
Test data sheets
Incubator with controlled lighting

Attachment 2

Algae Test Data Form

Date:

Study Identification:

Investigator:

Condition of thalli used: poor fair good

Time blades placed in release beaker:________________

Time spores removed from release beaker:______________

Temperature of spore solution:______________________

Spore motility check:_______________

Zoospore Concentration Check

Determine concentration with 5 counts:

1. _______________
2. _______________
3. _______________
4. _______________
5. _______________

Mean:____________ S.D.:____________

Mean *10,000*1.11= ______________spores/ml. This is the concentration of the zoospore release.

To determine volume of spores to deliver to test chamber:

12,750 spores/ml x ____________ml test solution/chamber = __________spores per test container.

________spores/chamber ÷ spore concentration_________spores/ml = _______ml/test container

______μl/test container

Temperature of spore solution: ______________________

Temperature of test containers: ______________________

Salinity of test containers (before/after): _________________

Time test containers stocked: ________________________

Incubation start time:______________________________

Test termination time: _____________________________

Comments:____

Attachment 3

Algal Toxicity Test Data Sheet

Test Start Date:__________ Test End Date:__________ SDS EC_{50}:

Start Time:__________ End Time:__________ Comments__________

Micrometer conversion factor:__________

Study Identification:__________

Sample ID	Germ	Non Germ	Length Measurements/Cell Number										Mean Length/Cell No.
			L1/C1	L2/C2	L3/C3	L4/C4	L5/C5	L6/C6	L7/C7	L8/C8	L9/C9	L10/C10	

Sample ID	Germ	Non Germ	Length Measurements/Cell Number										Mean Length/Cell No.
			L1/C1	L2/C2	L3/C3	L4/C4	L5/C5	L6/C6	L7/C7	L8/C8	L9/C9	L10/C10	

Date Prepared: May 5, 1990

Date Revised: June 10, 1994

EXTRACTION AND STORAGE OF POREWATER SAMPLES

1.0 OBJECTIVE

This protocol describes a procedure for extracting and storing porewater samples from marine, estuarine, or freshwater sediments for use in toxicity testing. A pressurized extraction device is used to force the pore water from sediment samples. This procedure may be performed in the laboratory or it may be performed at or near the site of sample collection since the sampling apparatus is portable.

2.0 PREPARATION

2.1 Description of the Porewater Extraction System

In earlier studies (Carr et al., 1989; Carr and Chapman, 1992) pore water was extracted from sediments using a device constructed of Teflon®. Since then, the design has been improved (Carr and Chapman, 1994) The polyvinyl chloride (PVC) extractors in current use are less costly to construct and easier to operate. This device has been used in numerous sediment quality assessment surveys (Carr, 1993; NBS, 1993; NBS, 1994a; NBS, 1994b; USFWS, 1992).

The extractor is constructed from a PVC compression coupling for 4" I.D. schedule 40 PVC pipe. These commercially-available couplings (Lascotite®) consist of a cylinder (25 cm height and 13 cm diameter) with threaded ends and threaded open compression nuts (Figure 1). The coupling is fitted with end plates cut from 7/16" thick PVC sheeting that are held in place by the threaded end nuts. The gaskets provided with the coupling are discarded and silicon O-rings are used to seal the top and bottom connections. The top end plate is fitted with a quick-release fitting where the pressurized air is supplied, and a safety pressure relief valve. Like the original Teflon® extractor, the bottom end plate (Figure 1) has several interconnected concentric grooves to facilitate flow of the pore water to the central exit port. A 5 µm polyester filter is situated between the bottom end plate and the silicon O-ring. Before a sediment sample is loaded, the bottom end nut is tightened in place by using the stationary bottom wrench (Figure 1) and a standard strap wrench.

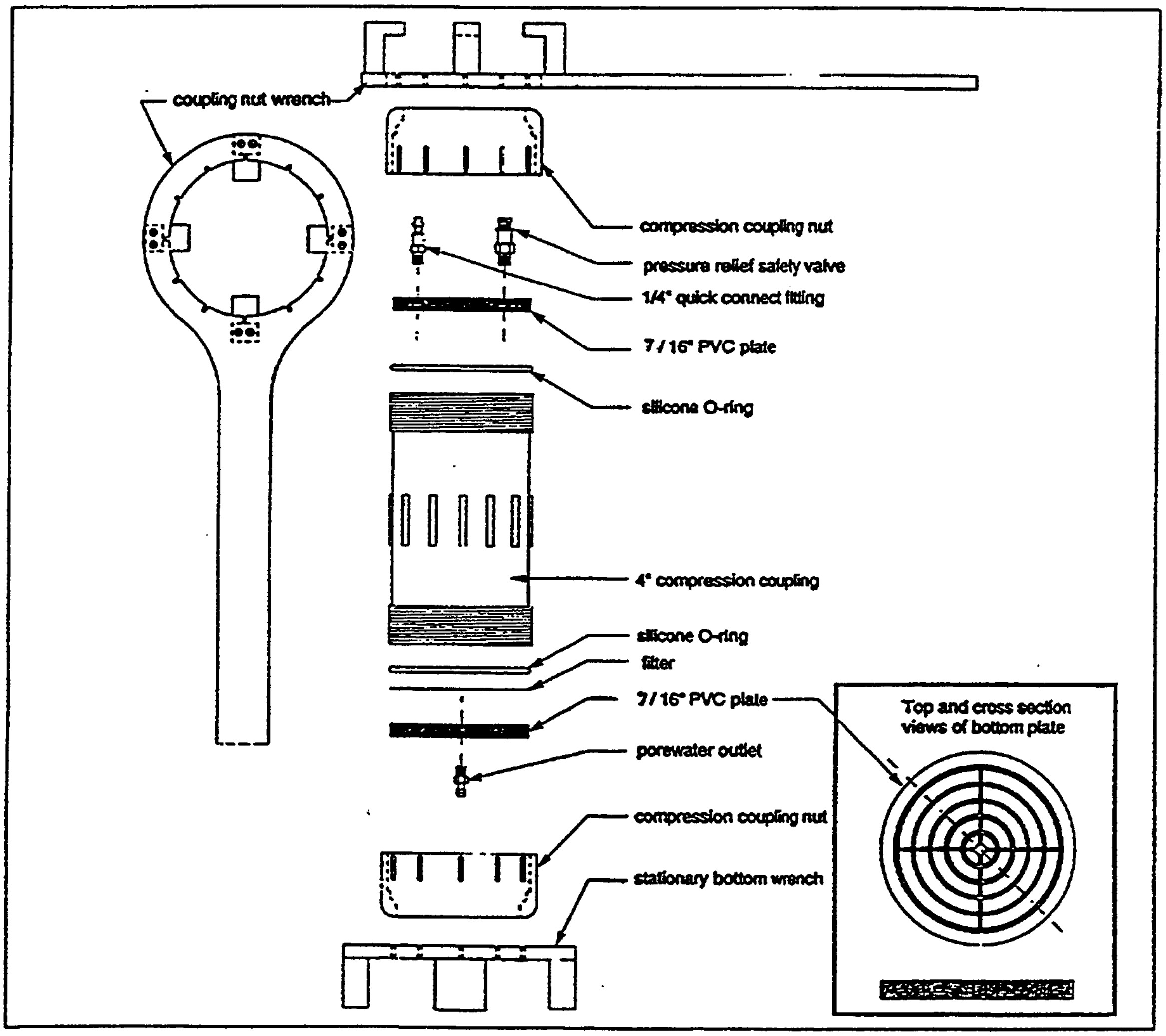

Figure 1. Sediment pore water squeeze extraction device.

The extractors are pressurized with air supplied from a standard SCUBA cylinder via a SCUBA first stage regulator which delivers air to a manifold with a valving system (Figure 2). With this system, multiple cylinders can be pressurized simultaneously, using the same SCUBA cylinder.

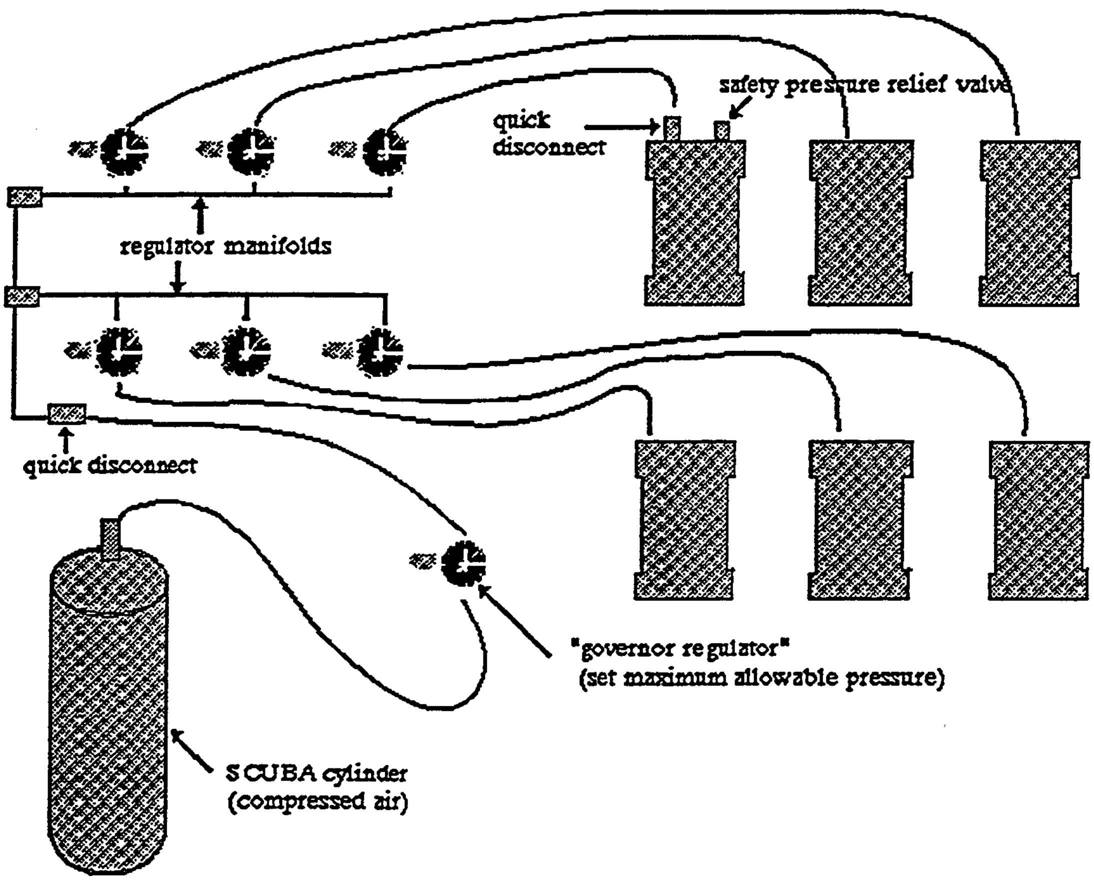

Figure 2. Schematic of sediment porewater pressure extraction system.

2.2 Equipment List

Supplies and equipment needed are listed in Attachment 1.

3.0 PROCEDURE

3.1 Sediment Collection and Storage Considerations

Generally, surficial sediment samples are collected for porewater extraction. A homogenate of the upper ~2-10 cm sediment may be collected by multiple cores or grabs at a particular sampling station. (Further details of sediment sampling procedures are not within the scope of this SOP.) One liter of sediment will typically provide 100-200 mL pore water. However, a larger volume of course sand sediments may be required since they contain less water, and a larger volume of fine clay sediments may be required since they are difficult to extract. The sample composites are kept in suitable containers (e.g., clean high density polyethelene containers or Zip-Lock® bags), labelled, and stored on ice, in a cooler, or in a refrigerator until the samples are delivered and processed. Pore water should be extracted from the samples as soon as possible because the toxicity of sediments in storage may change over time. A sample tracking system should be maintained for each sediment sample collected and porewater sample extracted. All manipulations made on samples are recorded on the Sample History Data Form (Attachment 2).

3.2 Load Extraction Cylinder

1. Assemble all parts of extraction cylinder except the top end compression coupling nut, top end plate and O-ring. Make sure filter is snugly in place beneath bottom O-ring (both over- and under-tightening will result in an improper seal). Place the extractor cylinder on the stand and positon an appropriately labelled porewater sample container (usually an I-Chem® amber 250 mL or 125 mL glass jar cleaned to EPA standards, with Teflon® lid liner) underneath the outlet.

2. Ensure that the sediment sample is homogenized, by shaking, stirring with a clean Teflon® or plastic spatula or spoon, or by both.

3. Transfer sediment from the sample container/bag to the extractor by pouring and/or using a clean Teflon® or plastic spatula or spoon. If necessary, particularly when extracting pore water from sandy or shelly sediments, the spatula may be used to compress the sample in the cylinder to eliminate channelization. The amount of sediment to be transferred will depend on the texture of the sample. The cylinder may be filled nearly full with a sandy sediment. However, when extracting pore water from a clay sediment, a relatively impermeable layer of compressed clay will eventually form on the filter, so that extraction of a large volume of clay sediment at once would take an extremely long time. When extracting pore water from extremely fine grained sediments, the cylinder should be less than one-third filled. If additional pore water is needed, this process can be repeated by removing the sediment including

sediment including removing or "peeling" the impermeable layer, and reintroducing more of the original sediment sample.

4. After sediment is loaded, the top end plate within the top compression coupling nut is installed . To tighten the top nut, the strap wrench and the coupling nut wrench (Figure 1) are used.

3.3 Porewater Extraction

After the extractor is sealed, a high-pressure hose is attached to the quick disconnect fitting on the top end plate, and the extractor is pressurized with air from a SCUBA tank. Pressure is controlled with a first-stage regulator on the SCUBA tank, an intermediate "governor" regulator, and final second stage regulators attached to a manifold that services multiple extractors (Figure 2).

1. Turn the SCUBA valve counter clockwise, pressurizing the first stage regulator and the intermediate-pressure hose (approximately 150 psi). An additional "governor" pressure regulator between the SCUBA tanks and the final second stage regulators which control pressure to the individual extractors should be set at maximum extractor pressure (~40 psi).

2. Ensure that all final pressure regulators are set to zero. Attach the hose from one of the pressure regulators on the pressure regulator manifold to the air inlet, using the quick disconnect fitting.

3. Slowly open the corresponding pressure regulator to a pressure of 5-10 psi. Check the first drops of porewater passing from the outlet for cloudiness. Occasionally, a small amount of sediment will pass through the porewater outlet, presumably around the filter. If this happens, wait until the pore water clears, discard the initial pore water collected, and continue.

4. Check the cylinder for leaks and if necessary tighten clamping nuts slightly.

5. As the flow of pore water decreases, pressure may be increased gradually to a maximum of 35-40 psi. When flow is less than or slows to less than 1-3 drops per minute, increase the pressure in 5-10 psi increments to maintain the flow. Allow the extraction to continue until sufficient pore water has been collected.

6. Disassemble the extractor, discard sediment, and rinse and wash appropriately all parts contacting sediment before placing a different sediment sample into the extractor.

7. Repeat these procedures until all available extractors are in use or until all sediment samples have been processed.

3.4 Centrifugation of Porewater Samples

Porewater samples extracted at this field station are usually stored frozen until tested. Under most circumstances, the porewater samples are centrifuged after they are collected and before they are frozen.

1. After collection, keep the porewater samples refrigerated or chilled on ice until they are centrifuged.

2. Transfer the pore water from the glass sample jar to an appropriate centrifuge bottle (e.g., polycarbonate). Centrifuge at ≥1200 *g* for 20 minutes. Return the centrifuged sample to a rinsed and labelled glass jar, taking care not to disturb any material that may have settled on the bottom/sides of the centrifuge bottle.

3. If multiple jars of pore water were collected from a single sediment sample, they should be composited after centrifugation and redistributed to the glass jars before testing or storage.

3.5 Storage of Porewater Samples

If the porewater samples are not to be used on the day of collection, they should be frozen for storage. Sufficient room for freeze expansion should be left in the jars (for example, 200 mL maximum sample in a 250 mL jar). If the volume needed for testing is known in advance, it is prudent to allocate only that specific volume plus a little excess (~10 mL) to each jar in order to conserve pore water (once thawed, the pore water cannot be refrozen and reused), and to simplify the volume measurements required for Water Quality Adjustment of Samples (SOP F10.12) performed the day prior to testing. Frozen porewater samples may be shipped with dry ice.

4.0 QUALITY CONTROL

A sample tracking system is maintained for each sediment sample collected and porewater sample extracted. All actions taken with that respective sample are recorded on the Sample History Data Form (Attachment 2). This information includes, but not exclusively, : a) the date of collection or receipt, b) the date of porewater extraction, c) the volume or number of jars (I-Chem® amber glass jars) of pore water collected, d) centrifugation information, if performed, e) date frozen and location (freezer no.), and e) date and jar no. thawed and used in which test. The Sample History Forms are kept in a three-ring binder at the same location where the samples are stored.

5.0 TRAINING

Persons who will perform this procedure should first read this SOP and then operate under the supervision of an experienced individual for at least one series of extractions.

6.0 SAFETY

The sediment and porewater samples handled may contain contaminants. Care should be taken to avoid contact with the samples. Protective gloves, glasses and clothing may be worn. Waste sediment should be properly disposed. SCUBA cylinders should be securely mounted before, during, and after use. The pressure limit (40 psi) of the extraction cylinders should not be exceeded. Before disconnecting any pressure hoses, ensure that the pressure has been released or that the controlling regulator has been closed. The pressure relief valves should be set to leak at just above maximum operating pressure, and they should be checked regularly to ensure that they are performing. Pressure relief valves should be disassembled and cleaned yearly.

7.0 ATTACHMENTS

Attachment 1. Required Equipment and Materials
Attachment 2. Sample History Form

8.0 REFERENCES

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National Biological Survey (NBS). 1994b. Toxicity testing of sediments from Boston Harbor, Massachusetts. Final report submitted to National Oceanic and Atmospheric Administration, 6 pp. + 10 tables and 4 attachments.

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Attachment 1

REQUIRED EQUIPMENT AND MATERIALS

To construct a sediment pore water extraction device:

1-PVC cylinder (center portion of 4" compression coupling)
2-PVC end nuts (ends of 4" compression fitting)
1-PVC top end plate (7/16" width)
1-PVC bottom end plate (7/16" width)
1-Quick disconnect brass air fitting
1-Pressure relief valve
1-Teflon® 1/8" npt male connector for exit port

To use a pore water extraction device:

1-Filter, polyester material, 5 µm pore size
1-Wooden stand (1 stand per 3 cylinders)
1-Custom wrench for 4" compression coupling end nuts
1-Custom wrench head attached to table
1-Plastic or Teflon® spatula or spoon
1-SCUBA cylinder
1-SCUBA regulator with high pressure gauge
1-SCUBA intermediate pressure hose (~10 ft length)
    with governor pressure gauge set to ~40 psi
1-Air pressure control manifold that includes:
    Final pressure regulator valves (several per manifold)
    Pressure gauges (1 per valve)
    Low pressure hose, 6' length (1 per manifold)

Other required supplies/equipment:

Sediment sample containers or bags
Pore water sample jars
Sample labels or labeling tape
Beakers
Deionized water (DI)
Wash bottles, 500 ml
Protective gloves, glasses, clothing
Pens, pencils, markers
Centrifuge and centrifugation materials
Refrigerator
Freezer

Attachment 2

SAMPLE HISTORY DATA FORM

Sample Designation: ________ Study Protocol: ________________________ Initials: ________

Date of acquisition: ____________________________ Sample type: ____________________

How acquired (refer to sample site data sheet number, if appropriate): ____________________

____

____

____

Initials	Date	Action Taken

This is NOT A CITABLE DOCUMENT
and is intended for reference only.

Date Prepared: March 14, 1991

Date Revised: May 17, 1994

WATER QUALITY ADJUSTMENT OF SAMPLES

1.0 OBJECTIVE

In order to perform toxicity tests with saline samples, all test and reference samples should be similar in salinity so that salinity is not a factor in survival of test organisms. Additionally, dissolved oxygen (DO) concentrations should be sufficiently high to ensure that low DO is not a source of stress to the test organisms. At the Corpus Christi field station, toxicity tests are performed using a variety of marine and estuarine organisms, including the sea urchin *Arbacia punctulata*, the polychaete *Dinophilus gyrociliatus*, the harpacticoid copepod *Longipedia* sp., and the red drum *Sciaenops ocellatus*. The aqueous samples tested may be pore water, different kinds of discharges and effluents, surface microlayer, or subsurface water samples that may range in salinity from 0-36‰. Although from test to test salinities used in the different toxicity tests may vary, the individual toxicity tests performed on a particular day are run at a single target salinity. Since initial salinities of the porewater or water samples to be tested commonly vary, they will require salinity adjustment to within 1‰ of the target salinity. Additionally, DO should normally be ≥80% saturation in all samples tested.

2.0 PREPARATION

2.1 Equipment and Labware

The supplies and equipment needed are listed in Attachment 1.

2.2 Source of Dilution Water

For samples lower in salinity than target salinity, concentrated brine (~100‰) is added to increase salinity. Concentrated brine is prepared by heating (to 35-40°C) and gently aerating filtered natural seawater (1 μm) to concentrate the salts by evaporation. For samples higher in salinity than target salinity, HPLC ultrapure sterile water (J.T. Baker® Cat. #JT4218-2) is added to decrease salinity.

3.0 PROCEDURES

The following describes the procedures required for the adjustment and determination of specific water quality parameters of a sample.

3.1 Preparation for Salinity Adjustment

1. Although fresh samples are routinely tested at the Corpus Christi field station, most of the samples tested are stored frozen in amber I-Chem® jars. If frozen, remove samples from freezer and allow them to thaw at room temperature or immerse them in a tepid water bath to thaw, ensuring that sample temperature does not exceed 25°C. The samples may be thawed the day of water quality adjustment (WQA) or may be transferred from the freezer to a refrigerator (4°C) the day before WQA and then completely thawed the following day. After thawing, allow the samples to come to room temperature. Generally, the samples should be maintained at the same temperature required for the toxicity test that will be conducted. The temperature requirement for most toxicity tests performed at this field station is 20±1°C, and room temperature should be maintained accordingly.

2. Turn bottled sample end over end a few times to mix thoroughly before measuring salinity. Using a salinity refractometer, measure salinity and record on Water Quality Adjustment Data Form (Attachment 2).

3. In order to make calculations for the salinity adjustment, the volume of the sample must be known. When porewater or other water samples are collected and transferred to amber jars for storage, they are commonly measured to an approximate volume (~110 mL, for example) prior to freezing. On the day of WQA, this volume should be recorded on the WQA data form for the respective samples. If the volume is unknown at this point, it should be measured using a graduated cylinder of appropriate size, and recorded on the data sheet.

3.2 Salinity Adjustment

3.21 Reducing the salinity of aqueous samples

Refer to the formulas below to calculate the volume of HPLC water needed to reduce the initial sample salinity to the target salinity. Add the volume calculated, mix the bottle thoroughly, check the salinity with a refractometer, and record the volume of HPLC water added as well as the final salinity.

(i) (target $^{\circ}/_{\infty}$ ÷ sample $^{\circ}/_{\infty}$) × sample vol. in mL = A
(ii) sample vol. - A = B
(iii) sample vol. ÷ A = C
(iv) B × C = volume of HPLC water to add

3.22 Increasing the salinity of aqueous samples

Refer to the formula below to calculate the volume of concentrated brine needed to increase the initial sample salinity to the target salinity. Add the volume calculated, mix the bottle thoroughly, check the salinity with a refractometer, and record the volume of brine added as well as the final salinity.

(i) ((target $^{\circ}/_{\circ\circ}$ - sample $^{\circ}/_{\circ\circ}$) × sample vol. in mL) ÷ (brine $^{\circ}/_{\circ\circ}$ - target $^{\circ}/_{\circ\circ}$) = vol. of brine to add

3.3 Dissolved Oxygen Adjustment

Measure and record DO and percent DO saturation of sample (SOP F10.13). Occasionally, a sample will have DO of less than 80% saturation. Any such samples should be gently stirred on a magnetic stirrer to increase the DO level above 80%. Record initial DO, the elapsed mixing time, and final DO in the comments section of the Water Quality Adjustment Data Form. (On the following day, DO should be rechecked and brought to >80% by stirring again if necessary before the toxicity test is performed.)

3.4 Other Water Quality Determinations

1. Measure pH (SOP F10.21) and record on the Water Quality Adjustment Data Form.

2. Measure and record ammonia concentration (SOP F10.4).

3. Measure and record sulfide concentration if required.

4.0 DATA COLLECTION

All raw data are entered on one standardized form, the Water Quality Adjustment Data Form (see Attachment 2) at the time the determinations or adjustments are made.

5.0 QUALITY CONTROL

A data form (Attachment 2) will be used to document all sample handling procedures for each sample. The person(s) recording data on the sheet will initial each sheet. Original data forms after completion will be stored in a three-ring file in the possession of the field station leader. Copies will be kept in the lab.

6.0 TRAINING

Personnel who will perform this task should first read this protocol and then operate under supervision during the preparation of at least two samples.

7.0 SAFETY

The NaOH solution used in the ammonia determination procedure is a highly caustic liquid. Care should be taken to avoid its contact with skin or clothing. Should such contact occur, quickly flush affected with water. A sink is present along the west wall of the dry lab, another is present along the east wall of the wet lab, and an eye flushing station is present in the northwest corner of the wet lab near the entrance door. The samples handled may be pore water, effluent, discharges, or other water samples that may contain contaminants. Care should be taken to avoid contact with the samples.

8.0 ATTACHMENTS

Attachment 1. Equipment List for Water Quality Adjustment
Attachment 2. Water Quality Adjustment Data Form

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ATTACHMENT 1

EQUIPMENT LIST FOR WATER QUALITY ADJUSTMENT

Graduated cylinders
Pipetters
Latex gloves
Magnetic stirrer and stir bars
10 M NaOH
Concentrated brine (See section 2.2 for preparation)
HPLC ultrapure sterile water (J.T. Baker® #JT4218-2)
Salinity refractometer
Dissolved oxygen meter
pH electrode, buffer solutions, and meter
Ammonia electrode, standard solutions, and meter
Sulfide electrode, standard solutions, and meter
Data sheets
Hand calculator

ATTACHMENT 2

WATER QUALITY ADJUSTMENT DATA FORM

STUDY PROTOCOL______________________________ INITIALS________________

SAMPLE DESIGNATION__________________ DATE______________________________

A. Salinity Adjustment:

Initial volume (mL)	________________
Initial salinity (°/₀₀)	________________
Vol. Baker® HPLC water added (mL)	________________
Vol. ____°/₀₀ brine added (mL)	________________
% of original sample (initial vol./final vol. x 100)	________________

B. Character of Sample (after salinity adjustment):

Final Volume (mL)	________________
Final Salinity (°/₀₀)	________________
pH	________________
Dissolved oxygen (mg/L)	________________
DO saturation (%)	________________
Total ammonia (mg/L)	________________
Sulfide (mg/L)	________________

COMMENTS____